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## ***Section 5:*** ***Control Strategies***

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## ENVIRONMENTAL FATE OF A GENETICALLY MARKED ENDOPHYTE IN GRAPEVINES

### Project Leader:

Blake Bextine  
Dept. of Entomology  
University of California  
Riverside, CA 92521

### Project Director:

Thomas A. Miller  
Dept. of Entomology  
University of California  
Riverside, CA 92521

### Consultant:

Frank Richards  
Yale University  
New Haven, CT 06520

### Collaborators:

Carol Lauzon  
Dept. of Biological Sciences  
California State University  
Hayward, CA 94542

David Lampe  
Dept. of Biological Sciences  
Duquesne University  
Pittsburgh, PA 19219

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### ABSTRACT

Symbiotic control employs symbiotic bacteria to deliver anti-pathogen compounds to disrupt transmission of the pathogen to new hosts. *Alcaligenes xylosoxidans denitrificans* (*Axd*), an insect and plant symbiotic bacterium, occupies same niche as the plant pathogen *Xylella fastidiosa* (*Xf*) which causes Pierce's disease. We determined the fate of genetically altered *Axd* (*RAxd*) after introduction into grapevines to assess its feasibility as a symbiotic control organism to control *Xf*. *RAxd*, which expresses a fluorescent protein (DsRed), was applied to grapevines by needle inoculation, foliar spray application, or soil drench. The plants were covered with insect-resistant screening, to exclude arthropods from test plants. *RAxd* were detected in stems of several grapevines 2 weeks post-inoculation from each inoculation type. The amount detected at 4 weeks post-inoculation declined, and *RAxd* was absent 6 weeks post-inoculation. *RAxd* was not detected in grape berries or soil samples collected around *RAxd* positive grapevines. This work demonstrated that transgenic *Axd* became established in grapevines in the field but did not thrive there. A limited lifespan of transformed *Axd* in grapevines would keep its population increase in check in that host plant. Re-inoculation of grapevines at 6 wk intervals would be sufficient to keep anti-pathogen products present. *RAxd* thrives in GWSS and citrus. Therefore, there is a good chance that GWSS would pick up the *RAxd* as an antimicrobial symbiont from nearby sources to render GWSS vector-incompetent.

### INTRODUCTION

Replacement therapy or symbiotic control employs symbiotic bacteria to deliver anti-disease compounds to target pathogens of plants to make vector insects unable to harbor the pathogen or to prevent a pathogen from being transmitted to healthy plants (1). *Alcaligenes xylosoxidans denitrificans* (*Axd*), was selected for further study and a fluorescent marker gene inserted. We followed the movement of genetically altered *Axd* (*RAxd*) in grapevines and in the vector insect, glassy-winged sharpshooter (GWSS), *Homalodisca coagulata*.

Regulatory and industry acceptance of this approach requires knowing the fate of *Axd* in various locations and in plants at different times of the year. Our current detection methods employ PCR (polymerase chain reaction) and fluorescence microscopy (3, 4). QRT-PCR provides a quantitative measure of bacteria in the samples, which is missing from existing methods. This is important because it allows determining optimum doses and timing for application of the delivery organism and its expression of anti-*Xylella* products.

Fluorescent protein gene markers are now commonly used in genetics and are considered environmentally benign since they are based on natural products. The bacterial transformation cassette was inserted with so-called jumping genes (mobile or transposable elements) originally identified in *Drosophila mauritiana* and called mariners (7). The mariner elements have had their jump mechanism removed (so the inserted gene will not be remobilized) and all antibiotic genes used for selection have been removed (so no antibiotic factors can be moved inadvertently to other bacteria). The resulting transgenic strains are very stable and grow readily in culture. Little or no mutation or reversion has been observed.

Since the marker genes were placed next to an open reading site that is designed to contain the future anti-*Xf* compound, the bacteria we are using now are nearly complete. In other words it is close to the final product. Thus, we can study the biology of the genetically altered vehicle bacterium, *RAxd*, and its behavior in the vineyard ecosystem.

We prefer to do this in commercial vineyards because the laboratory experiments are never fully indicative of behavior in the field. We chose widely separated locations and in California and more than one variety of grapevine to test. A top priority was to determine if the transgenic endophyte lodged in the grape berries or otherwise contaminated the product of the vineyards.

## OBJECTIVES

1. Track the movement of *Alcaligenes xylosoxidans denitrificans* (*Axd*) within plants with or without insect involvement and track movement in the environment.
2. Characterize transmission of *Axd* by glassy-winged sharpshooter (GWSS, *Homalodisca coagulata*).
3. Develop an application method for transgenic *Axd* into the xylem of grape plants for delivery of an anti-*Xylella* strategy.

## RESULTS

In July 2003, field sites were established at four locations in the state of California; Napa, Bakersfield, Temecula, and Riverside. At the Napa, Bakersfield, and Temecula sites, *RAxd* was applied to grapevines using 3 inoculation techniques; needle inoculation, foliar spray application, and soil drench. These plants were covered with insect-free screening, to exclude arthropods from test plants. Samples were taken throughout the growing season and processed. Grapevines at the Riverside field site were needle inoculated with *RAxd* and three concentrations of GWSS (0, 10, and 50) were placed on the plants to test the affect of GWSS feeding pressure on the translocation of *RAxd* in grapevines. We collected mature grapes and plant parts for analysis from grapevines at all four field sites.

### *Detection of RAxd in Grapevine Xylem: Napa Field Site*

Grapevines were inoculated 41 days prior to harvest. Pre-harvest grapevines xylem samples were collected three times (2, 4, and 6 weeks post-inoculation). Only single samples from 2 of 15 test plants tested positive for the presence of *RAxd* at 2 weeks post-inoculation. These positives were from plants treated by needle inoculation and soil drench. Two weeks later, only a single sample from the soil drench-treated plant tested positive. There were no positive samples collected 6 weeks after inoculation. No control plants tested positive for the presence of *RAxd* on any date.

### *Bakersfield Field Site*

Grapevines were inoculated 33 days prior to harvest. Pre-harvest grapevines xylem samples were collected two times (2 and 4 weeks post-inoculation). Multiple samples from 8 of 15 test plants tested positive for the presence of *RAxd* at 2 weeks post-inoculation. Of these *RAxd* positives plants, 3/5 from foliar spray, 2/5 from needle inoculation and 3/5 from soil drench. Two weeks later, only two plants from the foliar spray-treated grapevines tested positive. No control plants tested positive for the presence of *RAxd* on any date.

### *Temecula Field Site*

Grapevines were inoculated 43 days prior to harvest. Pre-harvest grapevines xylem samples were collected 3 times (2, 4, and 6 weeks post-inoculation). No samples on any collection date tested positive for the presence of *RAxd*.

### *Riverside Field Site*

Grapevines were inoculated 26 days prior to harvest. Pre-harvest grapevines xylem samples were collected two times (2 and 4 weeks post-inoculation). Only 10 samples collected 2 weeks after inoculation were positive for the presence of *RAxd*. Six of the positive samples were from grapevines with no GWSS included, while 4 of the positive samples were from grapevines with GWSS included. No significant differences in *RAxd* presence in grapevines could be attributed to the presence of GWSS ( $\chi^2=0.24$  df=1,  $p$  value=0.624).

### *Detection of RAxd in Soil. RAxd*

Detection of *RAxd* in soil. *RAxd* was not detected in soil samples collected from the base of any grapevines at any locations using the culture methods or RT-PCR.

### *Detection of RAxd in Grape Berries*

In grape cluster samples collected on the date of harvest (Napa Aug. 27, Bakersfield Sept. 3, Temecula Sept. 2, and Riverside Aug. 18), *RAxd* was not detected by RT-PCR in whole grape samples from any location. Furthermore, *RAxd* was not detected in dissected grape berry samples of flesh, veins, seeds, peduncle, or stem from any location.

### *Detection at the Time of Field Plot Destruction*

*RAxd* was not detected in grapevine, root, or soil samples at the time of removal.

**Table 1.** Detection of *RAxd* in grapevines from three field sites (2003).

Application Method		<i>RAxd</i> positive samples <sup>1</sup>				Berries <sup>4</sup>	During grapevine removal <sup>2</sup>	
		Weeks post-inoculation					Canes	Root <sup>5</sup>
		0	2	4	6 <sup>3</sup>			
Bakersfield	Foliar Spray	0	<b>3</b>	<b>2</b>	ND	0	0	0
	Needle Inoculation	0	<b>2</b>	0	ND	0	0	0
	Soil Drench	0	<b>3</b>	0	ND	0	0	0
	Control	0	0	0	ND	0	0	ND
Napa	Foliar Spray	0	0	0	0	0	0	0
	Needle Inoculation	0	0	0	0	0	0	0
	Soil Drench	0	<b>1</b>	<b>1</b>	0	0	0	0
	Control	0	0	0	0	0	0	ND
Temecula	Foliar Spray	0	0	0	0	0	0	0
	Needle Inoculation	0	0	0	0	0	0	0
	Soil Drench	0	0	0	0	0	0	0
	Control	0	0	0	0	0	0	ND

<sup>1</sup>Represents 6 samples from 5 grapevines per treatment per field site (n=30 per grapevine).

<sup>2</sup>Grapevines were removed >14 weeks after inoculations at all locations.

<sup>3</sup>ND = not determined.

<sup>4</sup>Berries were collected during final collection date.

<sup>5</sup>Root samples were taken only from *RAxd* treated vines.

#### 2004 Field Project

Data are not complete and will not be reported here.

#### CONCLUSIONS

Grapevine inoculations were made after 50-80 days following grapevine flowering at all locations. After flowering, both the xylem and the phloem begin to fill the fruit with fluid (6). Between 60 and 70 days after flowering, the xylem stops filling the fruit, and phloem contributes all fluid for the development of the fruit. This flow continues to 120 days after flowering which is the average number of days to fruit harvest. At all field sites, *RAxd* inoculations were made 26 (Riverside) to 43 (Temecula) days prior to harvest. Considering grapevine physiology, inoculations were made after the xylem ceased to contribute fluid directly to the fruit in all cases. Therefore, it was not surprising that *RAxd* was not found in fruit at any location because it is a xylem-associated bacterium (3).

The most probable explanation for the inability of *RAxd* to survive in grapevines after 4 weeks was its lack of competitive fitness associated with the transgenic organism. Xylem contains diverse and sometimes extensive communities of microbes (2). In greenhouse studies, a strain of EGFP protein-expressing *Axd* was introduced into seedlings of several plant species (3). In that study, the genetically marked bacterium moved readily within the xylem vessels of the plants and was recovered 10 months later. However, presence of a well-established microbial community may have restricted the growth and colonization of transformed *Axd*, ultimately leading to its demise. Chromosomally transformed organisms are commonly less fit than native bacterial species due to the cost of the genetic insert (5). A comparison of the genetically modified *Axd* to the native *Axd* showed that the transformed strain was less fit in laboratory cultures (Lauzon, unpublished data). Although the experiments were not designed to test the relative fitness of transgenic *Axd*, the bacterium's inability to persist longer than 4 weeks provides additional support for the theory of reduced fitness.

Given the ubiquitous nature of *Axd* and its ability to colonize several plant hosts, including grapevines, in the greenhouse (3), we expected it to persist longer in field-grown grapevines. Even so, viability of 4 weeks may offer a large enough window for the delivery agent in a symbiont control strategy to dispense the necessary anti-pathogen factors to negatively affect *Xf*. Additionally, re-application of the symbiotic control agent may be necessary. Additionally, reduced fitness offers an internal controlled mechanism that will guard against transformed *Axd* population spread and persistence in the environment or consumer products.

## REFERENCES

1. Beard, C.B., Cordon-Rosales, C., and Durvasula, R.V. 2002. Bacterial symbionts of the Triatominae and their potential use in control of Chagas disease transmission. *Annu. Rev. Entomol.* 47:123-141.
2. Bell, C. R., Dickie, G. A., Harvey, W. L. G., and Chan, J. W. Y. F. 1995. Endophytic bacteria in grapevine. *Can. J. Microbiol.* 41:46-53.
3. Bextine, B., Lampe, D., Lauzon, C., Jackson, B.C., and Miller, T.A. 2004. Establishment of a genetically marked insect-derived symbiont in multiple host plants. *Curr. Microbiol.* In Press.
4. Bextine, B., Lauzon, C., Potter, S., Lampe, D., and Miller, T.A. 2004. Delivery of a genetically marked *Alcaligenes* sp. to the glassy-winged sharpshooter for use in a paratransgenic control strategy. *Curr. Microbiol.* 48:327-331.
5. De Leij, F.A.A.M., Thomas, C.E., Bailey, M.J., Whipps, J.M., and Lynch, J.M. 1998. Effect of insertion site and metabolic load on the environmental fitness of a genetically modified *Pseudomonas fluorescens* isolate. *Appl. Envir. Microbiol.* 64:2634-2638.
6. Mullins, M.G., Bouquet, A., and Williams, L.E. 1998. The Biology of the Grapevine.
7. Rubin, E.J., Akerley, B.J., Novik, V.N., Lampe, D.J., Husson, R.N., and Mekalanos, J.J. 1999. *In vivo* transposition of mariner-based elements in enteric bacteria and mycobacteria. *Proc Natl Acad Sci.* 96:1645-1650.

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# PARATRANSGENESIS TO CONTROL PIERCE'S DISEASE: BIOLOGY OF ENDOPHYTIC BACTERIA IN GRAPE PLANTS AND BIOASSAY OF REAGENTS TO DISRUPT PIERCE'S DISEASE

**Project Leader:**

Blake Bextine  
Dept. of Entomology  
University of California  
Riverside, CA 92521

**Project Director:**

Thomas A. Miller  
Dept. of Entomology  
University of California  
Riverside, CA 92521

**Consultant:**

Frank Richards  
Yale University  
New Haven, CT 06520

**Collaborators:**

Carol Lauzon  
Dept. of Biological Sciences  
California State University  
Hayward, CA 94542

David Lampe  
Dept. of Biological Sciences  
Duquesne University  
Pittsburgh, PA 19219

Don Cooksey  
Dept. of Plant Pathology  
University of California,  
Riverside, CA 92521

**Reporting Period:** The results reported here are from work conducted from April 2003 to October 2004.

**ABSTRACT**

*Xylella fastidiosa* (*Xf*), which causes Pierce's disease (PD) in grapevines, is transmitted by the glassy-winged sharpshooter (GWSS). Symbiotic control employs symbiotic bacteria to deliver anti-pathogen compounds to disrupt transmission of the pathogen to new host plants. *Alcaligenes xylosoxidans denitrificans* (*Axd*) was identified as a potential agent for paratransgenesis because it inhabits the foregut of GWSS and the xylem of plants, as does *Xf*. In this report, we describe the relationship between *Axd* (the symbiont), *Xf* (the plant pathogen), GWSS (the insect vector), and host plants to develop a delivery strategy for symbiotic control. Additionally, disruption of *Xf*-transmission by GWSS was demonstrated using two reagents, a single chained antibody fragment and an antibiotic peptide.

**INTRODUCTION**

The glassy-winged sharpshooter (GWSS) is the principal vector of the xylem-limited bacterium *Xylella fastidiosa* (*Xf*), which causes Pierce's disease (PD) in grapevines. Limiting the spread of this pathogen by rendering GWSS incapable of pathogen transmission would control the disease.

Symbiotic control approaches have been developed to disrupt Triatomid transmission of *Trypanosoma cruzi* [3], to prevent colitis in mammals [4, 11], and to interfere with transmission of HIV [8]. Candidate microbes that live in close proximity to the pathogen in the vector insects and in host plant tissues would be ideal vehicles to control *Xf*.

*Alcaligenes xylosoxidans denitrificans* (*Axd*), originally isolated from the cibarium of GWSS, has been described as a non-pathogenic plant endophyte and a non-pathogenic soil-borne microbe [10, 12]. *Axd*, genetically marked with DsRed or EGFP protein, colonized the cibarium of GWSS for up to 35 days, the longest period tested [6]. *Axd* readily colonized the xylem vessels of several plants with citrus being the most hospitable to the bacterium. [5]

Two categories of anti-pathogen reagents, single-chained antibodies (scFV) and antibiotic peptides, were tested for activity against *Xf*. Screening of scFV uncovered an antibody fragment that was specific to *Xf* and may be specific to the PD-causing strain of *Xf*. Four toxic peptides were identified that inhibited the growth of *Xf*, but did not inhibit the growth of *Axd*.

**OBJECTIVES**

1. Identify relationships between *Axd* (the symbiont), *Xf* (the plant pathogen), GWSS (the insect vector), and host plants to develop a delivery strategy for symbiotic control.
2. Test the ability of anti-pathogens to disrupt *Xf* disease cycle.

**RESULTS*****Axd* Movement and Colonization within Host Plants**

In two trials, chrysanthemums (n=20) were needle inoculated with DsRed *Axd*, which contains a kanamycin-resistance gene. One week later phloem and xylem fluid samples were collected independent of one another using a Scholander pressure bomb [7]. The collection resulted in about 20-50 µl of phloem fluid and 100-150 µl of xylem fluid per stem. DNA was extracted from the remaining half of each phloem and xylem fluid sample from each plant using the Extract-N-Amp<sup>TM</sup> Plant kit (Sigma Aldrich, Steinheim, Germany). Presence of *Axd* was then determined using QRT PCR. The other half of each phloem and xylem fluid sample was inoculated into LB broth containing kanamycin and incubated for 48 h at 37°C. After the incubation period, bacteria were screened for red fluorescence using a MZ12 fluorescent microscope (Leica Microsystems Inc., Heerbrugg, Switzerland). Positive samples were confirmed by QRT PCR.

A higher proportion of xylem fluid samples tested positive for the presence of *Axd* than phloem samples in both trials: in trial 1 xylem 8/20, phloem 2/20 ( $\chi^2=4.8$ , 1df,  $p=0.0284$ ); in trial 2 xylem 15/20, phloem 8/20 ( $\chi^2=5.013$ , 1df,  $p=0.025$ ). In all

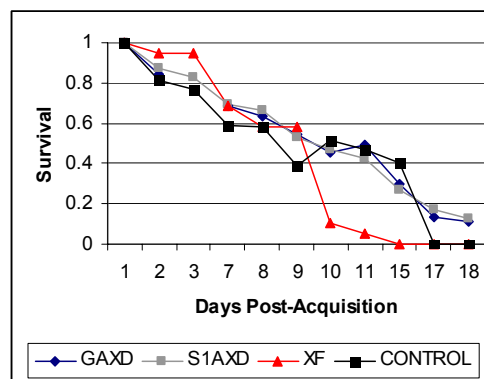
cases, positive phloem samples were detected only when the corresponding xylem samples was positive, whereas, most xylem samples were positive when phloem samples were negative. This indicated that positive detection of *Axd* in the xylem was due to actual presence of the bacterium; detection in phloem may have been due to contamination. Of the samples that tested positive, xylem samples contained 10X more cells on average than phloem although these values were not significant at the  $p=0.05$  level (Trial 1:  $F=0.911$ , 1df,  $p=0.368$ . Trail 2:  $F=3.123$ , 1df,  $p=0.092$ ). All plant samples which tested positive by RT PCR were confirmed by culturing followed by visualization under fluorescent microscopy.

### ***Movement of Axd into GWSS Populations***

After being exposed to an artificial feeding system containing DsRed *Axd* for 48h [6], 2 GWSS were marked with paint and placed on an individually caged chrysanthemum with 10 naïve GWSS for 2 weeks. At the end of this period, all GWSS were collected from the cage and analyzed for the presence of DsRed *Axd* by QRT PCR. In two trial, each with 10 replicates (10 individually caged plants), 81% of the test insects survived through the studies. In both trials, more than 57% of the surviving, previously “naïve”, GWSS tested positive for the presence of *Axd* (Trial 1, 51.2%; Trial 2, 64.3%). Therefore, through passive delivery of the symbiont in a finite period of time, more than ½ of the insects acquired the bacterium

### ***Effect of Axd or Xf on GWSS Biology***

Colonies of GWSS which were orally inoculated with DsRed *Axd*, wild-type *Axd*, S1 *Axd* (bacterium expressing an antibody), *Xf*, or no introduced bacteria (control) were maintained under laboratory conditions. Feeding ability, natural mortality, and dry weight post-mortem were compared between groups to determine if the presence of bacterium influenced any of these biological factors. In preliminary studies, mean g consumed after 5 was not significantly different for any of the 5 groups ( $n=20$ ,  $p<0.001$ ). Mortality rates of GWSS maintained in the laboratory were plotted over an 18 day period (Figure 1). Decline of the colony was consistent over time and no significant differences in mortality rates were detected ( $p<0.001$ ). Randomly, individual GWSS were collected from each colony, dried in a desiccating oven for 48 h, and weighed. The average weight of a dried GWSS was 0.01g and no significant differences in dry weight were revealed ( $p=0.7$ ). In two trials, 50 wild GWSS that were collected on the UCR campus were microinjected with different amounts either, DsRed *Axd* or  $H_2O$ . Bacteria were detected in the hemocoel; however, based on Chi-square analysis there were no significant differences in the mortality rates between the two groups.



**Figure 1.** Mortality rate in four GWSS colonies maintained in the lab oratory.

### ***Laboratory-Based Artificial Disease Cycle***

A simple and efficient transmission cycle was developed for the study of *Xf* transmission by GWSS which allowed collection of sufficient transmission data in 1 wk. Specific numbers of cells were detected both in plant tissue and within the insect vector by QRT PCR. *Xf* cells were scraped from a PD3 plate and suspended in sterile ½ strength PBS ( $OD^{600}=2.0$ ). Five cm sections of cut chrysanthemum stems were used for bacterial inoculations [6]. Five GWSS per 5 cm of stem were caged in snap cap vials for 48 h. After the acquisition access period (AAP), GWSS were placed on *Xf*-free chrysanthemums for 48 h, so that any detection of bacteria would be associated with transmission and not stylet contamination. Pairs of GWSS were transferred to sterile vials containing a fresh chrysanthemum stem cutting. The insects were exposed to a stem for an inoculation access period (IAP) of 48 or 96 h. DNA was extracted from the inoculation targets with the XNAR Extract-N-Amp kit (Sigma-Aldrich, St. Louis, MO) and PCR was run following a standard QRT-PCR protocol. Across 9 replicates using a 48h IAP, the mean transmission rate of *Xf* by GWSS was  $0.508 \pm 0.122$ , while the mean rate when given a 96h IAP was  $0.341 \pm 0.138$ . Using Chi-square analysis, these ratios were significantly different ( $\chi^2=16.281$ ,  $df=1$ ,  $p<0.001$ ). The lower rate associated with the longer IAP is probably due to the non-hospitable environment of the test plant stems.

### ***Interruption of PD Cycle***

Transmission of *Xf* from infected grapevine to healthy grapevine by GWSS was blocked by feeding GWSS on the plant-based AFS containing an *Xf*-specific antibody fragment (scFV S1) expressed in the coat of a M13 bacteriophage, between a 5 d AAP and the 5 d inoculation access period (IAP). At two concentrations of phage/antibody ( $10^{14}$  and  $10^{15}$ ) transmission of *Xf* was 0% ( $n=10$  and  $n=13$ , respectively), compared to 50% transmission in the control group ( $n=8$ ). Transmission of *Xf* was reduced when GWSS were fed Indolicidin (American Peptide Company, Inc., Sunnyvale, CA) between the AAP and IAP from 50% in the control group to 35% ( $n=14$ ) at  $100\mu\text{g/ml}$  and 7% ( $n=14$ ) at  $500\mu\text{g/ml}$ . These experiments are currently being replicated. While the rate of *Xf* transmission was higher than previously reported [1, 2, 9], we feel this is a fair assessment of the insects' ability to transmit.

## **CONCLUSIONS**

Several major biological associations were found which support the feasibility of symbiotic control to reduce transmission of *Xf* by GWSS:



1. Natural populations of GWSS are commonly found thriving on several citrus varieties.
2. *Axd* colonized and grew best in the citrus varieties tested.
3. *Axd* colonized the xylem vessels of test plants, the same tissue from which GWSS feed.
4. *Axd* passively moved through populations of GWSS.
5. *Axd* did **not** negatively affect GWSS.

Interestingly, *Axd* appears to mirror the host range of GWSS. Genetically marked *Axd* colonizes several host plants. This suggests that genetic modification does not interfere with the biology of *Axd*, which should enter into the insect-plant cycle and be transmitted along with the pathogenic bacteria target. While GWSS is the vector of greatest interest in California, two other native sharpshooters also transmit the vehicle bacterium, *Axd*, and several plants can serve as hosts.

In the laboratory, inhibition of *Xf*-transmission by GWSS was demonstrated using two different categories of reagents, a surface antibody fragment and an antibiotic peptide (Indolicidin). The antibody fragment was specific to *Xf*. In our trials the antibody fragment was being expressed in the coat of a phage, so the effects on transmission might be greater when the antibody fragment is expressed on the surface of *Axd*. Indolicidin inhibited *Xf* growth *in vitro*, but did not affect growth of *Axd*. Transformation of *Axd* to produce each/or both of these reagents is currently under way.

We concluded that *Axd* will be an effective delivery agent of a symbiont control strategy for combating *Xf*. GWSS readily acquired *Axd* from a plant source and this bacterium translocated and colonized a variety of plants tested. We have yet to determine the effect of the reagents on *Xf* in infected grapevines.

Previously, plant symptoms confirmed by ELISA or PCR detection were used to determine if transmission had occurred. Unfortunately, these systems require the bacterium to colonize and infect the host plant to determine transmission. If an infected plant is asymptomatic, important but less obvious transmission events may be missed. Our system removes the plant “unknowns” from the equation. However, we recognize the importance of actual plant infection as a measure of transmission importance, but suggest using the artificial disease cycle as an initial rapid measure of vector competence.

## REFERENCES

1. Almeida RPP, Purcell AH (2003) *Homalodisca coagulata* (Hemiptera, Cicadellidae) transmission of *Xylella fastidiosa* to almond. Plant Dis. 87:1255-1259
2. Almeida RPP, Purcell AH (2003) Transmission of *Xylella fastidiosa* to grapevines by *Homalodisca coagulata* (Hemiptera: Cicadellidae). J. Econ. Entomol. 96:264-271
3. Beard CB, Cordon-Rosales C, Durvasula RV (2002) Bacterial symbionts of the Triatominae and their potential use in control of Chagas disease transmission. Annu. Rev. Entomol. 47:123-141
4. Beninati C (2000) Therapy of mucosal candidiasis by expression of an anti-idiotypic in human commensal bacteria. Nat. Biotechnol. 18:1060-1064
5. Bextine B, Lampe D, Lauzon C, Jackson B, Miller TA (2004) Establishment of a genetically marked insect-derived symbiont in multiple host plants. Curr. Microbiol. In Press.
6. Bextine B, Lauzon C, Potter S, Lampe D, Miller TA (2004) Delivery of a genetically marked *Alcaligenes* sp. to the glassy-winged sharpshooter for use in a paratransgenic control strategy. Curr. Microbiol. 48:327-331
7. Bextine B, Miller TA (2004) Comparison of whole-tissue and xylem fluid collection techniques to detect *Xylella fastidiosa* in grapevine and oleander. Plant Dis. 88:600-604
8. Chang TL-Y, Chang CH, Simpson DA, Xu Q, Martin PK, Lagenaur LA, Schoolnik GK, Ho DD, Hillier SL, Holodniy M, Lewicki JA, Lee PP (2003) Inhibition of HIV infectivity by a natural human isolate of *Lactobacillus jensenii* engineered to express functional two-domain CD4. Proc. Nat. Acad. Sci. 100:11672-11677
9. Costa HS, Blua MS, Bethke JA, Redak RA (2000) Transmission of *Xylella fastidiosa* to oleander by the glassy-winged sharpshooter, *Homalodisca coagulata*. Hortsci. 35:1265-1267
10. Meade MJ, Waddell RL, Callahan TM (2001) Soil bacteria *Pseudomonas putida* and *Alcaligenes xylosoxidans* subsp. *denitrificans* inactivate triclosan in liquid and solid substrates. Microbiol. Lett. 204:45-48
11. Steidler L, Hans W, Schotte L, Neirynck S, Obermeier F, Falk W, Fiers W, Remaut E (2000) Treatment of Murine Colitis by *Lactococcus lactis* Secreting Interleukin-10. Science 289:1352-1354
12. Yang HL, Sun XL, Song W, Wang YS, Cai MY (1999) Screening, identification and distribution of endophytic associative diazotrophs isolated from rice plants. Acta Bot. Sinica. 41:927-931

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## EXPLOITING *XYLELLA FASTIDIOSA* PROTEINS FOR PIERCE'S DISEASE CONTROL

### Project Leaders:

George Bruening  
Dept. of Plant Pathology  
University of California  
Davis, CA 95616

Edwin Civerolo  
USDA, ARS, PWA  
San Joaquin Valley Agricultural Sciences Center  
Parlier, CA 93648

### Cooperators:

Abhaya M. Dandekar  
Department of Pomology  
University of California  
Davis, CA 95616

Goutam Gupta  
Biology Division  
Los Alamos National Laboratory  
Los Alamos, New Mexico

**Reporting Period:** The results reported here are from work conducted from October 15, 2003 to October 11, 2004.

### ABSTRACT

The *Xylella fastidiosa* (*Xf*) is the causal agent of Pierce's disease of grape. In previous work, we discovered, partially purified, and investigated the processing of the *Xf* protein MopB, which previously had been known only from the nucleotide sequence of its gene. The amino acid sequence of MopB, the uniform staining of *Xf* cells with fluorescent anti-MopB antibody and the abundance of MopB in total protein extracts of *Xf* cells suggest that MopB is the major outer membrane protein of *Xf*. As such, MopB is expected to participate in *Xf* colonization of grape xylem elements. We previously demonstrated that partially purified MopB binds to (xylem-rich) balsa wood or cellulose (filter paper) disks under conditions in which other proteins do not adhere. Here we report improvements in our MopB purification procedure and observations on adherence of MopB in *Xf* cells to cellulose disks under conditions that eluted other *Xf* proteins. A high (0.25mM) concentration of the cellulose fragment cellotetraose did not interfere with the binding of MopB to cellulose, suggesting that the binding reaction of MopB is not specific for cellulose. We exposed *Xf* cells or MopB to each of three fibrous polymer disks and to cellulose disks and observed similar adherence of MopB from both sources to all four polymer disk types. Thus, MopB appears to associate with porous materials generally when it is exposed to such materials in purified form or as *Xf* cells. The abundance and exterior exposure of MopB makes MopB an ideal target for Pierce's disease control strategies. We seek to develop soluble proteins with high affinity for MopB. We will apply, as an anti-*Xf* agent, a selected MopB-binding protein alone or as a chimera with a bacterial cell-inactivating peptide or protein. Our expectation is that expression of the anti-*Xf* protein, targeted to the xylem in grape rootstock, may result in the anti-*Xf* protein moving into and protecting the grafted scion. In this reporting period, experiments were initiated with the objective of creating a protein having high affinity for MopB. As a first step towards this objective, Project Scientist Paul Feldstein developed *E. coli* strains expressing surface elements of MopB protein, so that the experimentally compliant *E. coli* can be used to select proteins with high affinity for *Xf* MopB.

### INTRODUCTION

We have been investigating an abundant protein of *Xf*, MopB. We showed that MopB is the major outer membrane protein of *Xf* and is partly exposed on the outside of the bacterial cell. We purified MopB, prepared antibodies against it, and demonstrated an apparent affinity of MopB for cellulose. This last observation and the abundance of MopB suggested that MopB may participate in the initial attachment of *Xf* to the inner surface of the xylem vascular elements or in some other critical event in the initiation of infection leading to the development of Pierce's disease. Regardless of whether MopB is critical in this process, its location and prevalence support our contention that MopB is an ideal target for a *Xf*-specific bactericide or for a reagent that would coat and thereby inactivate *Xf* cells. Our strategy for creating a high-affinity MopB-binding protein is to begin with a protein that has evolved to bind tightly to the major outer membrane protein of *E. coli*, OmpA, and to convert the specificity of that protein from OmpA-binding to MopB-binding. The T2-like *E. coli* bacteriophage K3 has OmpA as its receptor. The K3 tail fiber adhesion gp38 is responsible for binding of bacteriophage K3 to OmpA in a reaction whose rate and irreversibility suggest a high-affinity association. Mutational conversion of gp38 from its natural receptor OmpA to other *E. coli* surface proteins has been demonstrated in several publications (Drexler et al., 1991, and references cited therein). In outline, our planned experimental steps for creating an anti-*Xf* protein are (i) replace the OmpA protein of *E. coli* with a protein that has MopB sequences displayed on the cell exterior, (ii) select variants of bacteriophage K3 that can infect the modified *E. coli* and also can bind to *Xf* cells, (iii) isolate the variant bacteriophage K3 gene *gp38* (expected to encode a MopB-binding gp38 protein), and (iv) genetically modify the MopB-binding gp38 to confer solubility and (in collaboration with the Gupta laboratory) possibly fuse the gp38 to a bactericidal peptide-encoding sequence. Step (v) will be the expression of a xylem-targeted version of the gp38 or gp38 fusion protein in rootstock and will be performed in collaboration with the Dandekar laboratory.

## OBJECTIVES

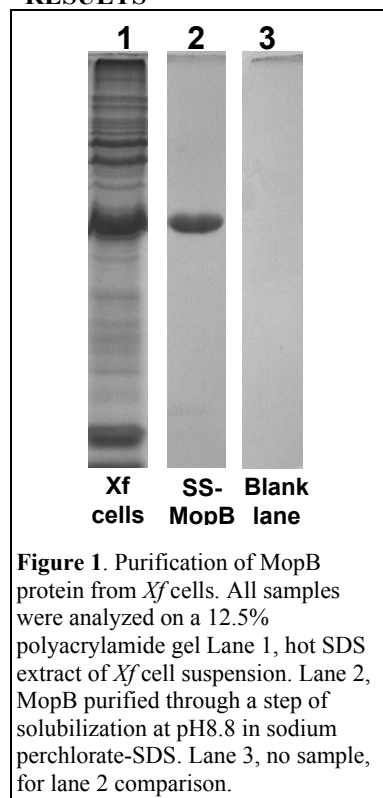
For period 15 Oct 2003 through 30 June 2004, previous project title “Roles of *Xylella fastidiosa* Proteins in Virulence”

1. To identify specific *Xylella fastidiosa* (*Xf*) protein(s) and determine their roles in virulence, particularly major outer membrane protein MopB
2. To develop strategies for interfering with *Xf* infection of grape and/or with development of Pierce’s disease

For period 1 July 2004 through 11 October 2004, new project title “Exploiting *Xylella fastidiosa* Proteins for Pierce’s Disease Control”

1. Discover or develop low molecular weight proteins with high affinity for portions of the MopB protein that are displayed on the *Xf* cell exterior.
2. Test MopB-binding proteins for their ability to coat *Xf* cells, for possible bactericidal activity, and for interference with disease initiation following inoculation of grape with *Xf*.
3. In collaboration with the Gupta laboratory, develop gene constructions for chimeric proteins designed to bind tightly to and inactivate *Xf* cells; express and test the chimeric proteins for their effects on *Xf* cells in culture.
4. In collaboration with the Dandekar laboratory, prepare transgenic grape expressing the candidate anti-*Xf* proteins; test the transgenics for resistance to infection by *Xf*

## RESULTS

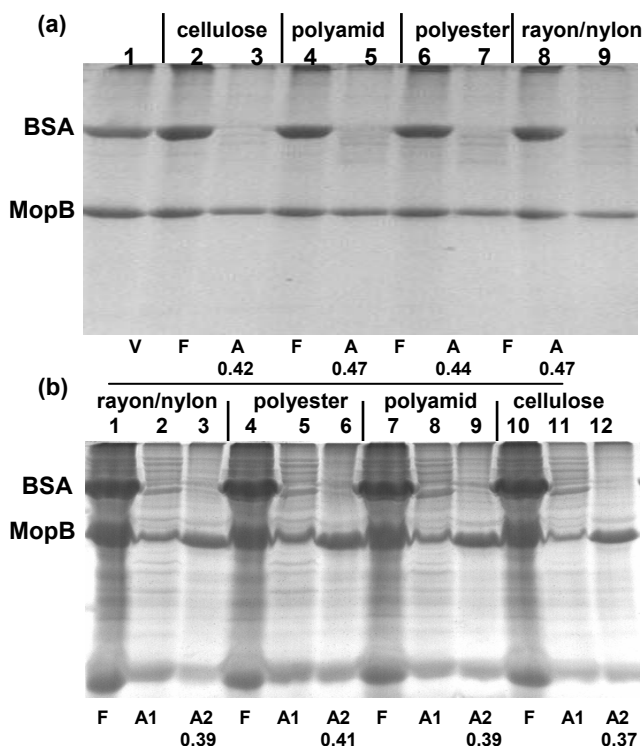


**Purification of MopB from *Xf* cells.** A dilute suspension of *Xf* cells scraped from plates is incubated at 30°C for 30 min in Tris-HCl-EDTA buffer pH 8.5 containing 8mg/mL SDS, 0.2μL/mL 2-mercaptoethanol. High speed centrifugation collects a precipitate (designated SP-MopB) that is highly enriched in MopB but includes substantial amounts of non-protein material from the *Xf* cells. The precipitate is dispersed into Tris-HCl-EDTA buffer, pH 8.8, containing 1.2M sodium perchlorate, 1mg/mL SDS, 10μL/mL 2-mercaptoethanol and is incubated at 30°C for 18hr. The supernatant after centrifugation at 50K rpm, 10°C for 20min is designated as the SS-MopB fraction. Sodium perchlorate reduces the solubilization of non-MopB proteins from SP-MopB preparations. The effective concentration of SDS is very low in SS-MopB due to the common ion effect with sodium perchlorate. SS-MopB, concentrated by centrifugal filtration, binds to porous polymer disks as described below.

**Preponderance of MopB in the *Xf* outer membrane.** *Xf* cells were washed with cold 1M perchloric acid to elute low molecular weight compounds. The cell suspension was assayed for DNA by the diphenylamine assay and for protein using the BCA reagent. The amount of DNA per stationary state cell is assumed to be  $2.7 \times 10^6$  base pairs. MopB appears to be 10-15% of the *Xf* cell protein, based on analyses such as those in Fig. 1. From these results, *Xf* cells have at least 80,000 MopB molecules per cell. We assume that the packing volume of MopB is similar to the packing volume derived from x-ray crystallography for the amino-terminal domain (residues 1-171) for *E. coli* OmpA, which crystallized as a 2.6nm diameter cylinder (Pautsch and Schutz, 1998). The diameter of a *Xf* cell is about 400nm. 80,000 molecules of hexagonally packed MopB would form a cylinder 400nm in diameter and almost 400nm high, accounting for more than 10% of the surface area of the 1000 to 5000nm long *Xf* cell.

**General association of MopB with porous substances.** We reported previously on the spontaneous association of MopB from solution with balsa wood (composed largely of xylem) and cellulose disks (filter paper). Other proteins, mixed with the MopB, did not absorb to balsa wood or cellulose. Fig. 2 reports our extension of this work to other porous polymeric materials of diverse chemical character. Cellulose, polyamid, polyester, and a rayon-nylon blend provided in approximately the same mass, all became associated with MopB, whether the MopB was supplied as partially purified protein in solution or as MopB in the outer membrane of *Xf* cells. Quantitatively, there was little variation in the extent of association among the polymers, all of which were exposed to the same NP-40 (non-ionic detergent) solution. Bovine serum albumin (BSA) was not absorbed by any of the porous polymer disks. Elution of polymer disks exposed to *Xf* cells in the presence of excess BSA was carried out in two stages. A mild elution (“A1” under the lanes in Fig. 2B), with neutral-pH SDS solution at 30°C, eluted most of the proteins not already removed from the polymer disks by the initial rinses with SCP buffer (“F” under lanes, Fig. 2B). Elution with hot, alkaline SDS-mercaptoethanol solution should remove all of the remaining proteins to the “A2” fractions. The A2 fractions contained about 40% of the MopB supplied to the disks in the initial incubation. However, only limited amounts of other *Xf* proteins remained after the A1 elution, i.e., to be eluted in the A2 fraction. We interpret these results as showing a tight association between MopB displayed on the outside of *Xf* cells and the polymers or a polymer-mediated precipitation of the MopB protein, which then could be released and/or solubilized only by exposure to hot, alkaline SDS solution. These results indicate no specificity of MopB for association with (or precipitation by) a specific polymer, so, unlike MopB itself, the polymer side of the MopB-polymer pair is not an attractive target for interfering with *Xf*-xylem interactions.

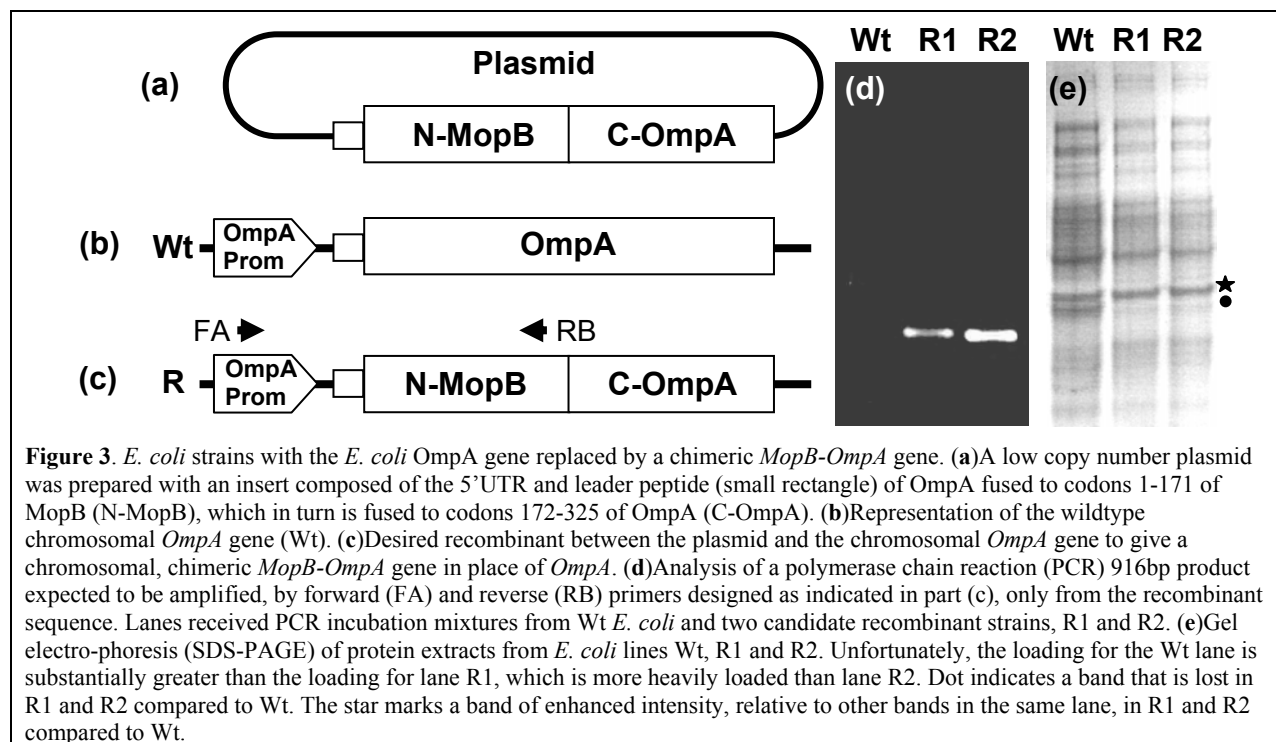
**Figure 2.** Polymer disk accumulation of *Xf* MopB from protein mixture and *Xf* cells. **(a).** A solution of SP fraction MopB and BSA was dispersed in 1x SCP, 1mg/mL NP-40. 8mm diameter disks were prepared from filter paper (2 disks, 19mg), polyamid (3 disks, 21mg), polyester (5 disks, 20mg), and 30% nylon, 70% rayon (3 disks, 19mg). 0.25mL of the BSA-MopB dispersion was dispensed into an empty vial (lane 1, V) and into vials containing polymer disks as indicated. The vials were incubated at room temperature for 2hr with orbital shaking at 100rpm. Free, unassociated material rinsed off with SCP: lanes 2, 4, 6 and 8 (F below lanes). Material eluted from polymer disks with alkaline hot SDS-mercaptoethanol solution: lanes 3, 5, 7 and 9 (A below lanes). **(b)** *Xf* cells were dispersed into 1xSCP, 1mg/mL NP-40 containing a great excess of BSA (150µg/mL). 0.25mL of the suspension was dispensed to vials containing polymer disks as indicated. Elution was in two stages: A1, SDS in SCP at 30°C and A2, hot SDS-mercaptoethanol at alkaline pH. Numbers under lanes indicate fraction of MopB band material in A and A2 fractions.



*E. coli* displaying MopB outer peptide loops. Attempted cloning and expression of the full *Xf mopB* gene in *E. coli*, including the *Xf MopB* promoter, were not successful. However, a system that included an inducible bacteriophage T7 RNA polymerase and T7 promoter driving the MopB-encoding sequence was adapted to create *E. coli* cultures generating low levels of MopB when induced with the gratuitous inducer IPTG. Intact *Xf MopB* accumulation may sicken *E. coli*, accounting for the low level accumulation. The Introduction describes in outline a strategy for creating a MopB-binding, anti-*Xf* protein. This strategy requires substitution of *E. coli* OmpA by a new outer membrane protein that portrays the characteristics of MopB on the surface of *Xf* cells. To this end, we created a chimeric MopB-OmpA construction in *E. coli* and subjected the cells to conditions designed to select cells in which recombination events resulted in the *E. coli* OmpA gene being replaced by the MopB-OmpA chimera (Fig. 3).

The predominant conformation of the OmpA protein as it resides in the outer membrane of *E. coli* probably has amino acid residues 1-171 inserted with 8 trans-membrane segments and four external loops (Singh et al., 2003). MopB can be cast in a similar conformation based on the crystallographic structure of OmpA and computer predictions of folding for OmpA and MopB. Our design for the chimeric MopB-OmpA gene retains the OmpA promoter and replaces only the 1-171 residue region of OmpA with the corresponding MopB sequence. Our rationale is that retaining the OmpA leader peptide, which targets the molecule to the outer membrane, and the OmpA carboxy-terminal portion, which includes the trans-periplasmic space sequences and the sequence that is inserted into the peptidoglycan layer, will result in a molecule that is more compatible with *E. coli* than an intact *MopB* gene would be.

The low-copy-number plasmid construction indicated in Fig. 3(a) encodes the desired chimeric molecule and the associated OmpA 5'UTR and leader peptide but lacks the OmpA promoter, so the chimeric protein should be expressed at a very low level, at the most, in transformed *E. coli*. The robust, highly recombination competent *E. coli* strain ER2738 was transformed with the Fig. 3(a) plasmid under the expectation that recombination events would replace the chromosomal *OmpA* gene [Fig. 3(b)] with sequences encoding the MopB amino-half molecule flanked by the OmpA leader peptide and carboxy-half OmpA sequences, creating the desired structure diagrammed in Fig. 3(c).



*E. coli* transformants displaying MopB sequences were selected using magnetic beads covalently coupled to anti-MopB IgG. Beads were plated on agar medium to recover colonies growing up from bead-selected cells. Pooled colonies were cultured, and the cells were exposed to the OmpA-specific bacteriophage K3 at a multiplicity of infection of 15 to deplete the population in cells still bearing OmpA. Fig. 3 provides evidence for the occurrence of the expected recombination events and for the production of the chimeric MopB-OmpA protein in amounts visible on a coomassie brilliant blue-stained gel [Fig. 3(d) and (e)]. The cells derived by these approaches agglutinate beads displaying anti-MopB IgG, providing evidence that some part of the MopB portion of the chimera, presumably the MopB outer loops, is displayed on the exterior of the *E. coli* cell.

## CONCLUSIONS

Based on results reported here and in previous progress reports, MopB is a highly suitable target for strategies designed to interfere with the ability of *Xf* to initiate infections leading to development of Pierce's disease. Our overall strategy for creating grape plants resistant to *Xf* is revealed by the four new objectives stated above in the Objectives section. Experimental steps (i), (ii) and (iii) outlined at the end of the Introduction reveal how we intend to satisfy new Objective 1. Results in Fig. 3 suggest that we have completed experimental step (i) and that we are ready to proceed to the selection of variant gp38 proteins capable of high affinity binding to MopB on the surface of *Xf* cells, i.e., experimental steps (ii) and (iii).

## REFERENCES

- Drexler, K., Dannull, J., Hindennach, I., Mutschler, B. & Henning, U. (1991). Single Mutations in a Gene for a Tail Fiber Component of an Escherichia-Coli Phage Can Cause an Extension from a Protein to a Carbohydrate as a Receptor. *Journal of Molecular Biology* **219**, 655-664.
- Pautsch, A. & Schulz, G. E. (1998). Structure of the outer membrane protein A transmembrane domain. *Nature Structural Biology* **5**, 1013-1017.
- Singh, S. P., Williams, Y. U., Miller, S. & Nikaido, H. (2003). The C-terminal domain of Salmonella enterica serovar Typhimurium OmpA is an immunodominant antigen in mice but appears to be only partially exposed on the bacterial cell surface. *Infection & Immunity* **71**, 3937-3946.

## Funding

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# CHARACTERIZATION OF NEONICOTINOIDS AND THEIR PLANT METABOLITES IN CITRUS TREES AND GRAPEVINES, AND EVALUATION OF THEIR EFFICACY AGAINST THE GLASSY-WINGED SHARPSHOOTER

## Project Leaders:

Frank Byrne  
Dept. of Entomology  
University of California  
Riverside, CA 92521

Nick Toscano  
Dept. of Entomology  
University of California  
Riverside, CA 92521

**Reporting Period:** The results reported here are from work conducted from July 2004 to October 2004.

## ABSTRACT

The toxicities of established and new members of the neonicotinoid insecticide class were assessed against the glassy-winged sharpshooter in topical application bioassays. All compounds were highly toxic to the insect. Clothianidin elicited its toxic response more rapidly than thiamethoxam and was 3-fold more toxic overall at the LD50 level. Clothianidin has been proposed as an active derivative of thiamethoxam, so it is important to establish the fate of these chemicals within plant systems that are likely to be treated for GWSS control.

## INTRODUCTION

The primary means of controlling the spread of Pierce's disease (PD) in California vineyards is through the elimination of its vector using insecticides. The glassy-winged sharpshooter (GWSS) *Homalodisca coagulata* feeds directly from the plant xylem system and, therefore, systemic insecticides are currently being evaluated on both citrus and grapes. Of the various classes of insecticide under consideration, the neonicotinoids, especially imidacloprid, have proven to be the most effective at suppressing GWSS populations. Imidacloprid (1-[(6-chloro-3-pyridinyl)methyl]-4,5-dihydro-N-nitro-1H-imidazol-2-amine) is a nicotinic acetylcholine receptor agonist that combines high potency with low mammalian toxicity and favorable persistence. As a systemic, seed, soil or foliar treatment, it has proved to be especially effective against a wide range of homopterous insect pests, including the GWSS. The success of imidacloprid in controlling GWSS is due largely to its excellent systemic properties. Systemic applications exploit the xylophagous feeding behavior of the insect, and thereby disrupt the transmission of PD and other *X. fastidiosa*-related diseases.

This project is an extension of a one-year project that was funded by the UC Pierce's Disease Research Grant Program. It will focus on the fate of imidacloprid and other neonicotinoid insecticides in citrus and grapevines, and the impact of these chemicals on GWSS. In a previous study, imidacloprid and two of its derivatives were shown to be highly toxic to GWSS adults (Byrne and Toscano, 2003).

The aims of this study are to determine the extent to which metabolites of neonicotinoids are formed in citrus trees and grapevines, and to determine their toxicological significance towards GWSS. The presence of insecticidal metabolites in xylem sap could contribute to the excellent persistence of imidacloprid treatments against sharpshooters. As well as maintaining the toxic pressure of the initial application, the metabolism of neonicotinoids to yield equally or more toxic metabolites may also account for the stability of this chemical class to resistance.

Of particular interest to us are thiamethoxam and clothianidin, which are being evaluated for use against citrus and grape pests. During the past year, it has been established that thiamethoxam is converted into clothianidin by insects and cotton plants (Nauen et al., 2003). This is an important finding, as it could have ramifications for the use of these products on grapes and citrus. When several products from the same class become available for pest management, it is important that their use be carefully monitored in order to circumvent potential resistance problems. The possibility that thiamethoxam is converted into clothianidin is, therefore, of concern when formulating management strategies based around the neonicotinoids. Receptor binding studies have suggested that thiamethoxam does not bind to the same receptor site as imidacloprid and so it has been proposed as a suitable product for alternation with imidacloprid because of the reduced resistance risk (Weisner and Kayser, 2000). Now that thiamethoxam has been shown to be a potential pro-insecticide, and clothianidin has been shown to bind to the same receptors as imidacloprid, new issues are raised about its suitability as a product for rotation with other neonicotinoids. This is an important reason for determining the fate of thiamethoxam in citrus and grapes.

## OBJECTIVES

1. Determine the metabolic fate of neonicotinoids within citrus trees and grapevines.
2. Determine the relative toxicities of neonicotinoids and their metabolites to the adult and egg stages of the GWSS.

## RESULTS

The toxicity of four neonicotinoid insecticides has been assessed for GWSS adults using a topical application bioassay (Table 1). Thiamethoxam, clothianidin and acetamiprid were all more toxic than imidacloprid. Clothianidin was

approximately 3-fold more toxic than thiamethoxam, and the dose-response was steeper as indicated by the higher slope. It was evident during these bioassays that the toxic effects of thiamethoxam were delayed compared with the other insecticides, suggesting that thiamethoxam may require activation to a toxic derivative within the GWSS.

**Table 1.** Toxicity of neonicotinoids to the GWSS in topical application bioassays.

Compound	LD50 (ng a.i. per insect)	95% FL	Slope	No. of insects
Imidacloprid	4.8	2-8	1.5 ±0.4	100
Thiamethoxam	2.6	2.0-3.3	1.4 ±0.3	200
Clothianidin	0.7	0.6-0.9	5.2±0.9	125
Acetamiprid	0.7	0.6-0.9	3.7±0.6	125

## CONCLUSIONS

In this study, we tested four neonicotinoids against the GWSS. Although there were differences in LD50s, all compounds were highly toxic. These results confirm that the newer neonicotinoids could have a place in GWSS management programs. We are currently investigating the fate of these chemicals in both citrus trees and grapevines. Establishing the potential for conversion of thiamethoxam into clothianidin is of particular importance if these chemicals are to be incorporated into management strategies.

## REFERENCES

- Byrne, F.J., and N.C. Toscano. 2003. Characterization of plant metabolites of imidacloprid in citrus trees and grapevines, and evaluation of their efficacy against the glassy-winged sharpshooter, *Homalodisca coagulata*.
- Nauen, R., Ebbinghaus-Kintscher, U., Salgado, V. L., and M. Kausmann. 2003. Thiamethoxam is a neonicotinoid precursor converted to clothianidin in insects and plants. *Pesticide Biochemistry and Physiology* 76: 55–69.
- Wiesner, P. and H. Kayser. 2000. Characterization of nicotinic acetylcholine receptors from the insects *Aphis craccivora*, *Myzus persicae*, and *Locusta migratoria* by radioligand binding assays: relation to thiamethoxam action. *Journal of Biochemical and Molecular Toxicology* 14: 221.

## FUNDING AGENCIES

Funding for this project was provided by the University of California Pierce's Disease Grant Program.

# EVALUATION OF RESISTANCE POTENTIAL IN THE GLASSY-WINGED SHARPSHOOTER USING TOXICOLOGICAL, BIOCHEMICAL, AND GENOMICS APPROACHES

## Project Leaders:

Frank J. Byrne  
Dept. of Entomology  
University of California  
Riverside, CA 92521

Nick C. Toscano  
Dept. of Entomology  
University of California  
Riverside, CA 92521

Brian A. Federici  
Dept. of Entomology  
University of California  
Riverside, CA 92521

**Reporting Period:** The results reported here are from work conducted from July 2004 to October 2004.

## ABSTRACT

Geographically distinct populations of GWSS differ in their toxicological responses to pyrethroid insecticides. We have shown that these different responses are unlikely to be caused by an esterase-mediated mechanism. The distributions of esterase activity in insects tested from Riverside and Redlands citrus orchards remained unchanged after selection with an LD50 dose of esfenvalerate.

## INTRODUCTION

We are using a multi-disciplinary approach to understand the biological and genetic mechanisms contributing to the toxicological differences between GWSS populations. This will allow us to determine whether the basis for decreased tolerance is due to target site changes or due to the selection of detoxification mechanisms. Whereas target-site modifications will only impact the pyrethroid class of insecticides, the selection of detoxification mechanisms are more critical due to their potential to confer cross-resistance to chemical classes that differ in their modes of action. In this first report, we describe selection experiments designed to test the potential involvement of esterases in conferring pyrethroid tolerance (Objective 2).

## OBJECTIVES

1. Monitor toxicological responses of geographically distinct populations of GWSS to pyrethroid insecticides
2. Measure biochemical activity of putative resistance-causing enzymes in these populations.
3. Clone and sequence the sodium-channel genes in GWSS populations differing in susceptibility to insecticides.
4. Perform microarray gene expression profiles in GWSS populations differing in susceptibility to insecticides to isolate novel genes involved in resistance.

## RESULTS

### *Bioassays*

Topical application bioassays (Byrne et al., 2003) have been conducted on Riverside GWSS adults to determine an LD50 for esfenvalerate. The LD50 was determined to be 0.75ng esfenvalerate per insect.

### *Selections*

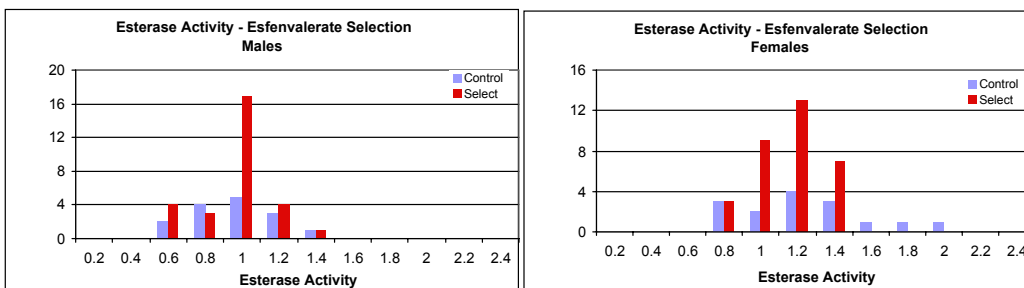
For selection experiments, insects were collected from the UC Agricultural Operations orchard in Riverside. Adults were treated with 0.75ng esfenvalerate by topical application. Esterase activity was measured in a subsample of insects taken before the bioassay, and in the survivors (at 48 hours) from the bioassay (Figure 1). Although there were differences in activities between males and females, there were no differences in activities attributable to selection by esfenvalerate.

In additional selection experiments, insects from Redlands and Riverside orchards were treated with 0 (controls), 0.075ng (sub-lethal) and 0.75ng (LD50) esfenvalerate per insect. Control and survivors at each treatment were used to prepare target RNA for gene expression profiling studies.

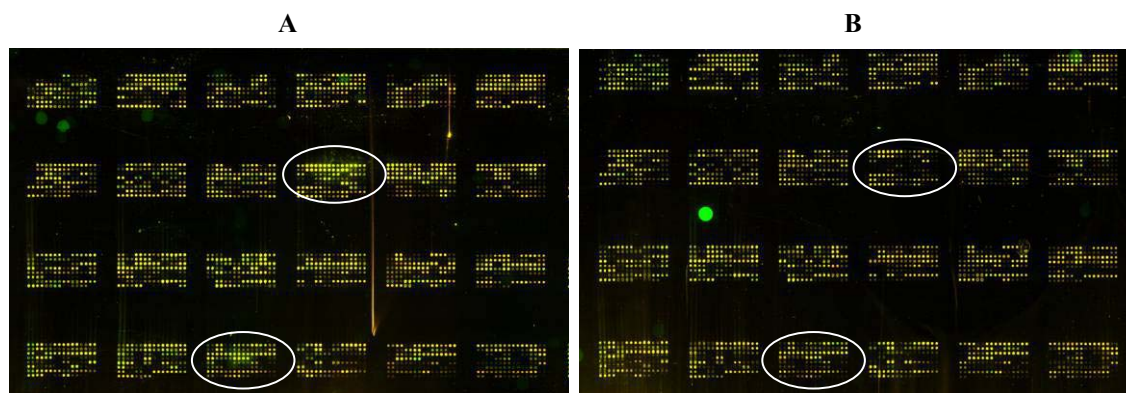
### *Microarrays*

PCR amplified inserts from 1,536 normalized library clones were spotted onto amino-silane coated glass slides. Each clone was spotted in side by side duplicate spots and the entire array was duplicated on each slide. Total RNA was isolated from two individual insects from each treatment for target preparation. Each total RNA was reverse transcribed and PCR amplified separately with Cy3- and Cy5-tagged dUTP. Slides were hybridized for 16 hours at 42°C on a Genomics Solutions GEN TAC<sup>®</sup> hybridization station and washed twice at medium stringency for 40 seconds. Each hybridization was repeated as a target dye swap. Slides were scanned on an Applied Precision Array Worx fluorescence scanner. Data is being evaluated using the Silicon Genetics GeneSpring program.





**Figure 1.** Distributions of esterase activity in adult male and female glassy-winged sharpshooters from a Riverside citrus orchard. Insects were treated topically with either acetone (Control) or 0.75ng esfenvalerate (Select), and esterase activity measured in survivors.



**Figure 2.** Scan data of microarrays hybridized to Cy3 labeled control target (green) and Cy5 labeled sub-lethal target (A) or LD50 target (B) (red). Circled results show obvious gene expression differences.

## CONCLUSIONS

In this study, we tested populations of GWSS from Riverside citrus orchards with 0.75ng esfenvalerate. This dose of esfenvalerate is the LD50 for the Riverside population when topically applied to the insect abdomen. Distributions of esterase activity revealed that there were no differences between the untreated insects and the treated survivors. These results suggest that esterases do not contribute directly to the toxicological differences between these populations. In addition, many and different gene expression changes occur in GWSS in response to sub-lethal and LD50 doses of esfenvalerate.

## REFERENCES

Byrne, F.J. and Toscano, N.C. 2003. Characterization of plant metabolites of imidacloprid in citrus trees and grapevines, and evaluation of their efficacy against the glassy-winged sharpshooter *Homalodisca coagulata*. Proceedings of the Pierce's Disease Research Symposium pp. 286-287, Coronado Island Marriott Resort, San Diego, California. Dec 8-11, 2003.

## FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

# FUNCTIONAL GENOMICS OF THE GRAPE-*XYLELLA* INTERACTION: TOWARDS THE IDENTIFICATION OF HOST RESISTANCE DETERMINANTS

## Project Leader:

Doug Cook  
Dept. of Plant Pathology  
University of California  
Davis, CA 95616

## Cooperators:

David Gilchrist  
Dept. of Plant Pathology  
University of California  
Davis, CA 95616

Andrew Walker  
Dept. of Viticulture and Enology  
University of California  
Davis, CA 95616

## Collaborators:

Choi, H.K., F. Goes da Silva, H. Lim, & J.E. Lincoln  
Dept. of Plant Pathology  
University of California  
Davis, CA 95616

A. Iandolino  
Dept. of Viticulture and Enology  
University of California  
Davis, CA 95616

**Reporting Period:** The results reported here are from work conducted from July 1, 2003 to June 30, 2004.

## ABSTRACT

We have used *in silico* mining of EST data and Real Time PCR to identify a set of *Xylella*-induced grape genes. Controlled time course analyses demonstrate that the genes are induced prior to symptom development, in coincidence with pathogen colonization. Analysis of field samples from grapes under a variety of biotic and abiotic stresses demonstrate that these genes are up-regulated in response to *Xylella* but not in response to the other pathogens assayed, including common viral, nematode and fungal pathogens, or by *Phylloxera* infestation or herbicide damage. By contrast, transcriptional responses similar to those observed in *Xylella*-infected tissues were observed in grapes under severe drought stress (in excess of normal field drought) and in plants where the vascular system had been blocked by damage from the grape cane girdler insect. These results are consistent with transcriptional regulation in response to insult within the vascular tissue of grape, but not to pathogen infection generally.

## INTRODUCTION

All organisms adapt to external stressors by activating the expression of genes that confer adaptation to the particular stress. For example, when exposed to conditions of heat or drought, genes for adaptation to heat and drought stress are up-regulated. Similarly, when a plant is exposed to a pathogen, numerous genes are induced including those that encode proteins involved in disease resistance. In the case of Pierce's disease, such genes are likely to include those coding for resistance to *Xylella* or to the insect vector.

Genomics technology offers an opportunity to monitor gene expression changes on a massive scale (so-called "transcriptional profiling"), with the parallel analysis of thousands of host genes conducted in a single experiment. In the case of Pierce's disease of grapes, the resulting data can reveal aspects of the host response that are inaccessible by other experimental strategies. Prior to carrying out transcriptional profiling, it is first necessary to (1) catalog the gene content of grapes by means of sequencing and bioinformatic analyses, and (2) develop gene-based arrays that allow the simultaneous monitoring of gene expression for >10,000 genes. Our research to date has contributed significantly in each of these areas. In May of 2004, the first Affymetrix gene chip was made available for public use, with ~15,000 *Vitis* genes represented. This gene chip has been developed based primarily on a collaboration between the Cook laboratory and researchers at the University of Nevada-Reno. With the arrival of the Affymetrix gene chip, we are poised to make a quantum leap in the identification of host gene expression in response to *Xylella fastidiosa*.

In addition to enumerating differences between susceptible and resistant genotypes of *Vitis*, the ongoing research will test a long-standing but largely untested hypothesis that pathogen-induced drought stress is one of the fundamental triggers of PD symptom development. The utility of this type of data will be to inform the PD research community about the genes and corresponding protein products that are produced in susceptible, tolerant and resistant interactions. Differences in the transcriptional profiles between these situations are expected to include host resistance and susceptibility genes, and thus provide the basis for new lines of experimental inquiry focused on testing the efficacy of specific host genes for PD resistance. It should be possible, for example, to determine the extent to which resistance responses in grapes are related to well-characterized defense responses in other plant species [e.g., Maleck et al., 2002; Tao et al., 2003; de Torres et al., 2003]. In addition to identifying candidate effectors of disease resistance, such knowledge would aid the development of testable hypotheses regarding susceptibility and resistance to *Xylella fastidiosa* in grapes.

Three co-lateral benefits from the identification of pathogen-induced genes are: (1) the promoters for such genes are candidates to control the expression of transgenes for resistance to Pierce's disease, (2) the protein products of induced genes may have roles in disease resistance, and (3) knowledge of host gene expression can be used to develop improved diagnostic assays for disease. In the first case, we are currently characterizing pathogen-responsive promoters, which would allow us to test candidate genes (the second case) for resistance phenotypes. In the third case, gene expression patterns can be used to develop so-called "molecular signatures" or "biomarkers" [MacNeil 2004] that are diagnostic of an organism's physiological status. Biomarkers are finding application in clinical medicine, where data on gene expression patterns are useful for characterizing disease states and improving clinical outcome [Alizadeh et al., 2001; Van't Veer et al., 2002; Ramaswamy et al., 2003]. In the case of Pierce's disease, the identification of early genes (i.e., genes expressed prior to the appearance of visible symptoms), and/or genes that are induced systemically in response to local infection, would greatly increase the reliability of disease diagnosis, which is currently prone to false negatives due to mis-sampling of locally-infected asymptomatic vines. At the same time, the identification of disease-related gene expression profiles would provide a novel measure of host response, and thus provide tools for basic Pierce's disease research applications.

## OBJECTIVES AND PRODUCTS OF THE RESEARCH

### *Completed objectives*

1. The public release of 61,203 EST sequences to the National Center for Biotechnology Information.
2. Development of a public, on-line relational database for analysis of the grape genome (<http://cgf.ucdavis.edu>).
3. Production of a public Affymetrix microarray, in collaboration with international researchers, available May 2004.

### *Ongoing Objectives*

4. Identify genes and gene pathways in susceptible *Vitis vinifera* correlated with *Xylella* infection: (a) identify *Xylella*-responsive genes in *V. vinifera*, (b) distinguish early from late gene expression, and (c) determine the correlation between drought stress and Pierce's disease.
5. Determine host genotype affects on gene expression in response to *Xylella* infection: (a) susceptible *Vitis vinifera* compared to resistant genotypes of *Vitis arizonica* and *Vitis aestivalis*, (b) comparison of pathogen-induced gene expression with gene expression triggered by salicylic acid and ethylene, and (c) analysis of gene expression in resistant and susceptible bulked segregants of *Vitis arizonica* X *Vitis rupestris*.
6. Development of Real Time PCR assay for routine monitoring of *Xylella*-induced genes under field, greenhouse and laboratory settings.
7. Isolation and characterization of *Xylella*-responsive plant promoters.

## RESULTS

### *Analysis of the Grape Transcriptional Response to Pathogen Challenge*

The results described below are based on the analysis of combined data sets generated under this project and that of our collaborators at the University of Nevada-Reno, and other members of the grape genomics community. In total, 40% of the 135K *V. vinifera* ESTs and 100% of the sequencing focused on Pierce's disease originated from this project.

### *In silico Identification of Xylella-induced Genes in Vitis vinifera*

We have identified 31 genes that appear to be up-regulated in response to infection by *Xylella fastidiosa*. The analysis, which involved construction of a correlation matrix and 2-dimensional hierarchical clustering, was based on EST frequency in various tissues with or without *Xylella* infection. The most abundant contig (7061) shares homology with a stress-related RNA from Arabidopsis, although the function is unknown in any system. Interestingly, this gene is up-regulated in infected plants, prior to symptom development, making it a top candidate for an early and sensitive marker of Pierce's disease. Other genes in the list have homology to proteins implicated in signaling during disease resistance, while others have been identified as pathogen responsive, or have been implicated in plant-insect interactions. After confirmation of the *Xylella*-specific transcription of such contigs (see Real Time PCR assays, below) we initiated the isolation of the promoters from these genes from genomic DNA libraries. The potential application of such promoters to drive *Xylella*-induced and/or tissue specific expression of transgenes is planned as a topic of a future grant proposal.

### *Development of Real-Time PCR for Gene Expression Analyses and Disease Diagnosis*

Detailed analysis of transcriptional responses will require methodical analysis by means of microarray gene expression studies, which we initiated in July 2004 under a one-year renewal to this project. At the same time, the current list of putatively *Xylella*-induced genes may provide leads for further analysis by means of Real Time PCR.

Real Time PCR has three primary uses for Pierce's disease research: (1) It can be used as an alternative to pathogen-based assays for disease diagnosis. For example, the identification of host genes that are expressed early and systemically could provide a significantly more reliable test for PD infection. This "biomarker" strategy is gaining increasing use for human medicine. (2) Real Time PCR assays offer a useful point of comparison for data from *in silico* analysis of gene expression (i.e., from statistical analysis of EST data) and for confirming results for key genes identified in Affymetrix microarray experiments. (3) Real Time PCR of differentially expressed host genes can provide a convenient research tool for investigators in need of a sensitive measure of host response.

Based on the *in silico* analysis, described above, four *Xylella*-induced genes, a constitutively expressed control *Vitis* gene, and a bacterial gene, were selected to develop a multiplex PCR assay. This "dual-diagnosis" system may have potential as a tool for disease diagnosis.

#### ***Isolation of Pathogen-induced Promoters***

DNA probes were developed based on the *Xylella*-induced genes and used to screen high-density filters of *Vitis vinifera* genomic DNA libraries. Clones were isolated, fingerprinted to confirm relatedness, and analyzed by PCR and sequencing to verify that they contained the genes of interest. A shotgun sequencing strategy is being used to obtain the complete sequence of each clone and promoter constructs are being made to test in transient and stable transformation assays. Gene fusions will include reporter proteins to monitor temporal and spatial patterns of transcription (e.g., green fluorescent protein and  $\beta$ -glucuronidase) and candidate pathogen resistance proteins that may protect grapes against *Xylella* infection.

#### **CONCLUSIONS**

To date we have identified several genes of *Vitis vinifera* that are up-regulated in response to *Xylella* infection. Ongoing research will identify larger sets of grape genes expressed in response to this pathogen and provide the basis for biotechnological approaches to dealing with Pierce's disease.

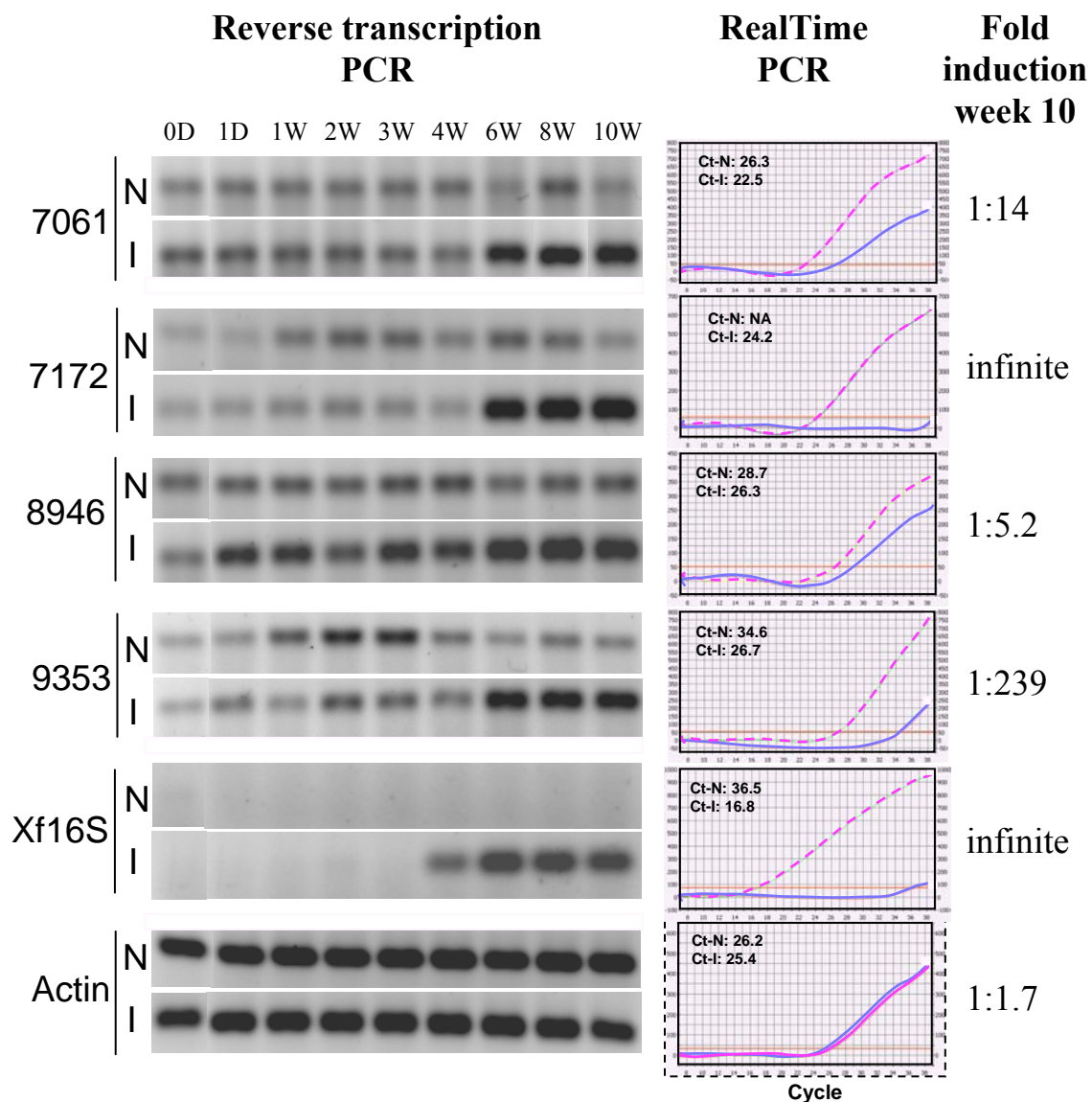
How will these technologies help in solving Pierce's disease? *In the short term* they will (1) yield improved genetic tools for breeding resistance to Pierce's disease (for example single nucleotide polymorphism "SNP" and simple sequence repeat "SSR" genetic markers currently available from our web site "<http://cgf.ucdavis.edu>"), (2) provide gene-promoters that are an essential, but currently unavailable, tool for effective genetic engineering in grapes, and (3) potentially provide the basis for more reliable detection of the pathogen based on Real Time PCR using a "biomarker" strategy. (4) *In the long term*, transcriptional profiling will identify candidate genes and gene pathways that may confer resistance to the pathogen (*Xylella fastidiosa*) and/or to the insect vector (Sharpshooter leaf hopper) and it will allow testing of long-standing hypotheses such as the relationship between host response to drought and host response to *Xylella*. Other strategies, such as reverse genetics and analysis of natural genetic variation for host responses, will be required to establish a causal role for candidate genes.

#### **REFERENCES**

- Alizadeh, A.A., et al. (2000) Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 403:503-511.
- MacNeil, J.S. (2004) Better Biomarkers for the Diagnostics Labyrinth. *Genome Technology* July/August, 24-33.
- Maleck, K., et al. (2001) The transcriptome of *Arabidopsis thaliana* during systemic acquired resistance. *Nature Genetics* 26:403-410.
- Ramaswamy, S., Ross, K., Lander, E.S. and Golub, T.R. (2003) A molecular signature of metastasis in primary solid tumors. *Nature Genetics* 33:49-54.
- Tao, Y., et al. (2003) Quantitative nature of *Arabidopsis* responses during compatible and incompatible interactions with the bacterial pathogen *Pseudomonas syringae*. *The Plant Cell* 15:317-330.
- de Torres, M., Sanchez, P., Fernandez-Delmond, I., and Grant, M. (2003) Expression profiling of the host response to bacterial infection: the transition from basal to induced defence responses in *RPM1*-mediated resistance. *The Plant Journal* 33:665-676.
- Van't Veer, L.J., et al. (2002) Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 415:530-536.

#### **FUNDING AGENCIES**

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**Figure 1. Monitoring of PD-induced genes using conventional reverse transcriptase-PCR and Real Time PCR.** Leaf tissue was sampled from growth chamber-grown plants at nine time points (0, 1d, 1w, 2w, 3w, 4w, 6w, 8w, 10w: d-day, w-week) after inoculation. *Xylella* up-regulated genes identified from in silico analysis are 7061, 7172, 8946, and 9353. Actin serves as a constitutively expressed control. *Xf16S* = *Xylella fastidiosa* 16S gene. N; Non-inoculated, I; Inoculated with *X. fastidiosa*.

## CONTROL OF PIERCE'S DISEASE THROUGH DEGRADATION OF XANTHAN GUM

### Project Leader:

Donald A. Cooksey  
Dept. of Plant Pathology  
University of California  
Riverside, CA 92521

### Cooperator:

Neal L. Schiller  
Division of Biomedical Sciences  
University of California  
Riverside, CA 92521

### Researchers:

Rosina Bianco  
Dept. of Plant Pathology  
University of California  
Riverside, CA 92521

Seung-Don Lee  
Dept. of Plant Pathology  
University of California  
Riverside, CA 92521

Korsi Dumenyo  
Dept. of Plant Pathology  
University of California  
Riverside, CA 92521

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### ABSTRACT

*Acinetobacter johnsonii* GX123, a *Xylella* gum-degrading endophyte was co-inoculated with *Xylella fastidiosa* strain Texas in oleander plants to determine its efficacy as a biocontrol agent in preliminary experiments. Symptoms appeared in both plants inoculated with *X. fastidiosa* alone and plants co-inoculated with the endophyte. However, symptoms were more severe and appeared earlier in plants inoculated with *X. fastidiosa* than in those co-inoculated with the endophyte. *A. johnsonii* GX123 seems to be a promising candidate to control *X. fastidiosa*. Experiments using a sequential strategy of inoculating the *Xylella* gum-degrader endophyte prior to *X. fastidiosa* are ongoing and its effects on symptom expression are still under investigation.

### INTRODUCTION

Pierce's disease (PD) of grapevine and other leaf scorch diseases caused by *Xylella fastidiosa* (*Xf*) are associated with aggregation of bacteria in xylem vessels, formation of a gummy matrix, and subsequent blockage of water uptake. In the closely-related pathogen, *Xanthomonas campestris* (*Xc*), xanthan gum is known to be an important virulence factor (Katzen et al, 1998), probably contributing to bacterial adhesion, aggregation, and plugging of xylem. The published genome sequence of *Xf* (Simpson et al, 2000; Bhattacharyya et al, 2002; Van Sluys et al, 2003) revealed that this pathogen also has genes for producing an exopolysaccharide with a very similar structure to that of xanthan gum. In PD, this *Xylella* gum is likely to contribute to plugging of the grapevine xylem (Keen et al, 2000) and possibly to the aggregation of the bacterium in the mouthparts of the glassy-winged sharpshooter. Because of its importance as an industrial thickener and emulsifier, xanthan gum synthesis and degradation have been extensively studied (Becker et al, 1998). Bacteria that produce xanthan-degrading enzymes have been isolated from soils using enrichment techniques with xanthan gum as the sole carbon source (Sutherland 1987; Ruijsenaars et al, 2000).

The purpose of this project is to identify bacteria that produce xanthan-degrading enzymes to target this specific virulence factor of *Xf*. This approach has the potential to significantly reduce the damage caused by PD in grapes and potentially in other hosts of *Xf* such as almond and oleander. If the gum is important in the aggregation of the pathogen in the insect vector, then our approach may also reduce the efficiency of transmission of PD. Our first approach will be to develop endophytic bacteria that produce these enzymes in the xylem of grapevines, but another approach is to engineer grape plants to produce these enzymes. Through the cloning and characterization of genes encoding xanthanases and xanthan lyases we will facilitate possible efforts to transform grapevines to produce these enzymes.

Previously, we used modified xanthan gum that mimics *Xylella* gum from a *Xc* mutant as the sole carbon source for enrichment culture from infected grapevines and oleanders. The *Xylella* gum biosynthetic operon in the *Xf* genome is different than the one in *Xc* from which the commercial xanthan gum is obtained. Since it is not feasible to produce *Xylella* gum for our studies from the slow-growing *Xf*, we genetically modified a strain of *Xc* to produce a modified xanthan gum that is predicted to have the same chemical structure as that from *Xf*. This was accomplished by deleting the *gumI* gene from the biosynthetic operon. Over 100 bacterial strains were initially recovered from enrichment experiments, and 11 were subsequently confirmed to effectively degrade *Xylella* gum. These strains were then tested for cellulase activity. Degradation of the cellulosic backbone of the gum polymer would be desirable, but we do not want enzymes that recognize and degrade plant cellulose. One particular strain (GX123) with high gum-degrading activity but no cellulase activity isolated from oleander was identified as *Acinetobacter johnsonii* (*Aj*), and characterized in more detail. In vitro, growth and biofilm production by GX123 were enhanced by *Xylella* gum as a substrate and by cells of *Xf* added to a minimal medium. The gum was degraded rapidly during log-phase growth of this endophyte, and viscosity was reduced almost to non-detectable levels. GX123 colonized stems and leaves of oleander systemically ( $10^4$ - $10^5$  cfu/g of plant tissue 20 days after inoculation), and systemic colonization was enhanced by co-inoculation with *Xf*. The effect of using GX123 as an endophyte to reduce the ability of *Xf* to produce disease symptoms in oleander was studied.

## OBJECTIVES

1. Characterize xanthan-degrading enzymes from endophytic bacteria isolated from grape
2. Explore applications of naturally-occurring endophytic bacteria that produce xanthan-degrading enzymes for reduction of Pierce's disease and insect transmission
3. Clone and characterize genes encoding xanthan-degrading enzymes for enzyme overproduction and construction of transgenic endophytes and plants

## RESULTS

### *Co-inoculation of the Xylella Gum-degrader Endophyte and X. fastidiosa in Oleander Plants*

GX123 was co-inoculated with *Xf* strain Texas in 3 different cultivars of oleander in the green house: White, Single Red and Betty. At the same time, controls were inoculated with GX123 alone, *Xf* alone or PBS buffer. Four plants were used per inoculation condition and per cultivar, totaling 48 plants obtained commercially. The appearance of symptoms was checked at approximately monthly intervals. Chlorotic mottling along the edges of leaves (Purcell et al, 1999) started to appear approximately in the eighth month after the inoculations, slowly developing into generalized chlorotic mottling and dried tissue (Table 1). The oleander cultivars White and Single Red were the first ones to show symptoms, while the cultivar Betty started to show symptoms 12 months after the inoculations. For all the cultivars, symptoms appeared in both plants inoculated with *Xf* and plants co-inoculated with the endophyte. However, the severity of the symptoms was less for the plants co-inoculated with the endophyte than for the plants not co-inoculated (Figures 1-3). Symptoms were more severe and appeared earlier in plants inoculated with *Xf* than in those co-inoculated with GX123 (Table 1 and 2). One year after being inoculated with *Xf* alone all the plants infected by *Xf* (positive result in ELISA test) showed symptoms, while one year after co-inoculations only 75% of the plants infected by *Xf* showed symptoms (Table 3). On the other hand, one year after inoculations *Xf* was detected in infected plants ( $10^5$ - $10^6$  ufc/g of plant tissue), while GX123 was not detected, showing a probable need for re-inoculation of the endophyte for a long term survival or a different strategy of introducing the biocontrol endophyte.

**Table 1.** Severity of the symptoms in oleander plants, regardless of the cultivar, inoculated with *X. fastidiosa* strain Texas alone or co-inoculated with GX123; 12 plants total per inoculation condition per month sampling.

Months	<i>X. fastidiosa</i> strain Texas				<i>X. fastidiosa</i> strain Texas/GX123			
	8	10	12	14	8	10	12	14
(+)	2	0	2	3	3	1	2	3
+	3	1	0	0	2	2	3	0
++	2	3	4	1	0	4	4	2
+++	0	3	3	4	0	0	0	5
AD	0	0	0	1	0	0	0	0
D	0	0	0	2	0	0	0	0

(+) chlorotic mottling along the edges of a few leaves; + chlorotic mottling along the edges of many leaves evolving into a uniform chlorotic mottling; ++ chlorotic mottling of many leaves, starting to wrinkle and dry; +++ chlorotic mottling of many leaves and zones of dead tissue (dried, straw color), smaller leaves; AD many dried leaves, plant almost dead; D plant dead.

**Table 2.** Number of symptomatic plants after inoculation with *X. fastidiosa* strain Texas alone, co-inoculated with GX123, GX123 alone or PBS buffer; 12 plants total per inoculation condition per month sampling.

Months	<i>X. fastidiosa</i> strain Texas	<i>X. fastidiosa</i> strain Texas/GX123	GX123	PBS
8	7	5	0	0
10	7	7	0	0
12	9	9	0	1
14	11	10	0	1

**Table 3.** Symptomatic plants and ELISA results after 1 year of inoculation; 12 plants total per inoculation condition.

Inoculations	<i>X. fastidiosa</i> strain Texas	<i>X. fastidiosa</i> strain Texas/GX123
Symptomatic plants	9	9
Positive ELISA for <i>X. fastidiosa</i>	9	12





**Figure 1.** Oleander 'White' after 1 year of inoculation with *X. fastidiosa* strain Texas.



**Figure 2.** Oleander 'White' after 1 year of co-inoculation with *X. fastidiosa* strain Texas and GX123.



**Figure 3.** Oleander 'White' after 1 year of inoculation with GX123.

#### ***Sequential Inoculation of the Xylella Gum-degrader Endophyte and X. fastidiosa in Oleander Plants***

To examine the effect of different strategies to introduce the *Xylella* gum-degrader endophyte to control *Xf* in plants, GX123 was inoculated in oleander plants (cultivar white) prior to *Xf*. Sequential inoculation of *Xf* was done 20 days after GX123 was inoculated in the same point when the titers of GX123 were already around  $10^4$ - $10^5$  cfu/g of plant tissue. This experiment is still ongoing and symptoms have not developed yet, consequently the effect on disease expression is still unknown.

#### **CONCLUSIONS**

The *Xylella* gum-degrader endophyte *Acinetobacter johnsonii* GX123 colonized plants and delayed symptoms of infected oleander plants in preliminary experiments. It is a potential candidate as a biocontrol agent for *Xylella fastidiosa*, and therefore a promising tool to fight Pierce's disease.

#### **REFERENCES**

- Becker, A., Katzen, F., Pühler, A., Ielpi, L. 1998. Xanthan gum biosynthesis and application: a biochemical/genetic perspective. *Appl. Microbiol. Biotechnol.* 50:145-152.
- Bhattacharyya, A., et al. 2002. Whole-genome comparative analysis of three phytopathogenic *Xylella fastidiosa* strains. *Proc. Natl. Acad. Sci. USA* 99:12403-12408.
- Katzen, F., Ferreira, D. U., Oddo, C. G., Ielmini, M. V., Becker, A., Pühler, A., Ielpi, L. 1998. *Xanthomonas campestris* pv. *campestris* gum mutants: effects on xanthan biosynthesis and plant virulence. *J. Bacteriol.* 180:1607-1617.
- Keen, N. T., Dumenyo, C. K., Yang, C.-H., Cooksey, D. A. 2000. From rags to riches: insights from the first genomic sequence of a plant pathogenic bacterium. *Genome Biology.* 1:1019.1-1019.3.
- Purcell, A. H., Saunders, S. R., Henderson, M., Grebus, M. E., Henry, M. J. 1999. Causal role of *Xylella fastidiosa* in oleander leaf scorch disease. *Phytopathol.* 89:53-58.
- Ruijsenaars, H. J., Hartmans, S., Verdoes, J. C. 2000. A novel gene encoding xanthan lyase of *Paenibacillus alginolyticus* strain XL-1. *Appl. Environ. Microbiol.* 66:3945-3950.
- Simpson, A. J. G., Reinach, F. C., Arruda, P., Abreu, F. A., et al. 2000. The genome sequence of the plant pathogen *Xylella fastidiosa*. *Nature* 406:151-159.
- Sutherland, I. W. 1987. Xanthan lyases – novel enzymes found in various bacterial species. *J. Gen. Microbiol.* 133:3129-3134.
- Van Sluys, M. A., de Oliveira, M. C., et al. 2003. Comparative analyses of the complete genome sequences of Pierce's disease and citrus variegated chlorosis strains of *Xylella fastidiosa*. *J. Bacteriol.* 185:1018-1026.

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## PARATRANSGENESIS TO CONTROL PIERCE'S DISEASE: TOXIC PEPTIDES AGAINST *XYLELLA*

### Project Leader:

Donald A. Cooksey  
Dept. of Plant Pathology  
University of California  
Riverside, CA 92521

### Researcher:

Ludmila Kuzina  
Dept. Plant of Pathology  
University of California  
Riverside, CA 92521

### Project Director:

Thomas Miller  
Dept. of Entomology  
University of California  
Riverside, CA 92521

### Collaborators:

David Lampe  
Biology Dept.  
Duquesne University  
Pittsburgh, PA 19219

Carol Lauzon  
Dept. of Biological Science  
California State University  
Hayward, CA 94542

Blake Bextine  
Dept. of Entomology  
University of California  
Riverside, CA 92521

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### ABSTRACT

The use of symbiotic bacteria in insects to disrupt pathogen transmission is a new approach to disease control. *Alcaligenes xylosoxidans denitrificans* bacterium was isolated from the mouthparts of wild glassy-winged sharpshooter and was chosen to be the first candidate for delivery products that inhibit *X. fastidiosa*. To find an appropriate agent for control of Pierce's disease, 90 antimicrobial peptides (AMPs) derived from a combinatorial peptide library (in addition to 59 screened previously from different sources) were tested for activity on 11 *X. fastidiosa* and 3 *Alcaligenes* strains. Forty four peptides showed potent antimicrobial activity against all strains studied. Six antimicrobial peptides (in addition to 4 found last year) were selected with toxicity to *X. fastidiosa* but not against *Alcaligenes* as a candidates for engineering of the sharpshooter's symbiont. More detailed studies of minimum inhibitory concentrations of these peptides were conducted. The Glutathione s-transferase gene fusion and *trc* expression systems are being developed to express individual AMPs *in vitro*.

### INTRODUCTION

*Xylella fastidiosa* causes of Pierce's disease (PD), an important disease of grapevines in the United States. Because of the mobility and vector capacity of glassy-winged sharpshooter (GWSS), PD has become a great concern to grape production in California. One promising method for long-term *X. fastidiosa* control is limiting pathogen spread by rendering GWSS vector-incompetent. Paratransgenesis (Beard et al. 2001), which is the genetic alteration of bacteria carried by insect is currently being developed to deliver pathogen toxic substances that would inhibit *X. fastidiosa* and reduce disease transmission.

Traditional antibiotics are natural or chemically synthesized small molecules that can selectively kill or stop growth of bacteria. A second type of antibiotics called antimicrobial peptides (AMPs) are produced by organisms including bacteria, plants, insects, birds, amphibians, and mammals (Cammue et al. 1992, Casteells et al. 1993, Nayler et al. 1989, Schroder 1999). These compounds interact directly with target bacterial membranes, but can do so with a receptor-like specificity, and can act via both membrane ion pore formation and by preventing cell wall formation (Maloy and Kari 1995). Because AMPs are "gene-based", they can be produced directly at the location where they are needed and their synthesis can potentially be regulated by using appropriate gene promoters. For example, the antimicrobial peptide MSI-99, an analog of Magainin 2, was expressed via the chloroplast genome to provide inhibition of growth against *Pseudomonas syringae* pv *tabaci*, a major plant pathogen (DeGray 2001). A combinatorial libraries represent a vast new source of molecular diversity for the identification of potential lead antimicrobial and antifungal compounds (Blonde and Lohner 2000, Jing et al. 2003). A combinatorial peptides are significantly shorter than other AMPs isolated from various biological sources. An amphipathic structure may allow this peptide to penetrate deeper into the interfacial region of membranes, leading to local membrane destabilization (Jing et al. 2003).

Use of symbiotic bacteria to deliver gene-based product is a new strategy of disease control. We demonstrated previously the expression of *Bacillus thuringiensis* toxin Cyt1A in the symbiotic bacterium *Enterobacter gergoviae* isolated from the gut of the pink bollworm (Kuzina et al. 2002). Bextine et al. (2004) used the expression of a red fluorescent protein (dsRed) by *Alcaligenes* (*Axd*) to study the colonization of the cibarial region of the GWSS. Genetically transformed symbiotic bacteria have been used to control the pathogen that caused Chagas disease (Beard et al. 1992, Beard et al. 2001, Durvasula et al. 1997).

### OBJECTIVES

The overall goal of this project is to genetically transform symbiotic bacterium of the glassy-winged sharpshooter to produce toxic substances that would inhibit or kill *X. fastidiosa* and reduce disease transmission.

1. Identify toxic peptides effective against *X. fastidiosa* but non-toxic to *Alcaligenes*, selected symbiotic bacterium.
2. Design and construct genes encoding indolicidin and other peptides.
3. Develop a transformation system for expression of indolicidin .
4. Construct a transport cassette for secretion of indolicidin into *Alcaligenes*.

## RESULTS

During the reporting period, we have screened an additional 90 antimicrobial peptides derived from a combinatorial library for activity on 11 *X. fastidiosa* and 3 *Alcaligenes* strains. *Axd* was isolated from the mouthpart of wild captured GWSS by Carol Lauzon. We found that 44 AMPs showed potent antimicrobial toxicity against all strains studied. Six AMPs were found with activity toward *X. fastidiosa* and non-toxic to *Alcaligenes*. These 6 peptides (along with 4 these screened last year) were more extensively examined for effective inhibitory concentration to *Xylella* and toxicity to *Alcaligenes* and *E. coli* as a target organism (Table 1). Blake Bextine studied the ability of GWSS to transmit *X. fastidiosa* to naive grapevine seedlings by oral delivery one of several antimicrobial peptide - indolicidin at 2 concentration: 100 µg/ml and 500 µg/ml. *X. fastidiosa* transmission rates were reduced from 50% in the control group, to 35% with the 100 µg/ml concentration and 7% with the 500 µg/ml concentration when GWSS were exposed to indolicidin prior to inoculation access. Therefore, indolicidin was chosen to be the first candidate for the development of gene-cassette. Artificial gene(s) to code indolicidin were designed and constructed for expression in *E. coli*. cDNA-encoding this peptide was amplified by PCR with incorporation of a *SalI* restriction site and/or *BamHI* and *EcoRI* restriction sites. We are using the Glutathione s-transferase gene fusion system (GST) (Pharmacia Biotech, Inc) and *trc* expression system (Invitrogen Co.) to express individual peptides. The GST gene fusion system is an integrated system for the expression, purification and detection of fusion proteins produced in *E. coli*. A pTrcHisTOPO expression kit provides a highly efficient, rapid cloning strategy for direct insertion of *Taq* polymerase-amplified PCR product into a plasmid vector for expression in *E. coli*. No ligase, post-PCR procedures, or PCR primers containing specific sequences were required. We transformed competent cells of *E. coli* DH5λ and TOPO by pGEX and pTrcHisTOPO vectors containing indolicidin gene. Several transformants were selected using LB medium containing ampicillin at 50 µg/ml (Sigma) and currently are being examined for production of indolicidin with and without IPTG.

**Table 1.** Toxicity of antimicrobial peptides to *X. fastidiosa*, *Alcaligenes*, and *E. coli* strains

Peptide	Range of MICs (µg/ml) to <i>X. fastidiosa</i> <sup>a</sup>	<i>Alcaligenes sp.</i> <sup>b</sup>	<i>E. coli</i> <sup>c</sup>	Source
1. Indolicidin	16-64	-	-	APS <sup>d</sup>
2. PA2	32-128	-	-	NCSU <sup>e</sup>
3. PA6	32-64	-	-	NCSU
4. PA7	32-64	-	-	NCSU
5. DCR1	16-32	-	-	TPIMS <sup>f</sup>
6. DCR2	8-16	-	-	TPIMS
7. DCR3	32-64	-	-	TPIMS
8. DCR4	16-32	-	-	TPIMS
9. DCR5	16-32	-	-	TPIMS
10.DCR6	8-16	-	-	TPIMS

<sup>a</sup> – MICs of the antimicrobial peptides to eleven *X. fastidiosa* strains studied

<sup>b</sup> – Activity of AMPs to *Alcaligenes xylosoxidans denitrificans* 134, 135, and 136 is negative

<sup>c</sup> – Activity of AMPs to *E. coli* DH5λ and TOPO is negative

<sup>d</sup> – American Peptide Company, Sunnyvale, CA

<sup>e</sup> – North Carolina State University, Raleigh, NC

<sup>f</sup> – Torrey Pines Institute for Molecular Studies, San Diego, CA

## CONCLUSIONS

The 10 antimicrobial peptides were found with toxicity to 11 *X. fastidiosa* strains isolated from grape, oleander and almond, but not against the glassy-winged sharpshooter gut bacterium *Alcaligenes xylosoxidans denitrificans*. We consider these AMPs as a candidates for use as reagents in delivery vehicle for paratransgenesis: Indolicidin, a 13-residue peptide-amide, isolated from the cytoplasmic granules of bovine neutrophils (Selsted 1992); 3 pescidins, isolated from the mast cells of aquacultured fish (Silphaduang and Noga 2001); and 6 peptides derived from a combinatorial peptide library (Blonde and Lohner 2000) (Table 1). *Alcaligenes* will be engineered to produce a peptide(s) toxic substance that would inhibit *X. fastidiosa* and reduce disease transmission. To develop a transformation system to express peptide(s) in *E. coli* first, we are using the Glutathione s-transferase gene fusion and *trc* expression systems. We got several ampicillin resistant transformants which are being studied for production of indolicidin. Artificial genes of other peptides are being designed for expression and secretion by *E. coli* and *Alcaligenes* as well.

## REFERENCES

- Beard, C.B., Mason, P.W., Aksoy, S., Tesh, R.B., Richards, F.F. 1992. Transformation of an insect symbiont and expression of a foreign gene in the Chagas disease vector *Rhodnius prolixus*. Am. J. Trop. Med. Hyg. 46:195-200
- Beard, C.B., Dotson, E.M., Pennington, P.M., Eichler, S., Cordon-Rosales, C., and Durvasula, R.V. 2001. Bacterial symbiosis and paratransgenic control of vector-borne Chagas disease. Int. J. Parasitol. 31:621-627
- Bextine, B., Lauzon, C., Potter, S., Lampe, D and Miller, T. 2004. Delivery of a genetically marked *Alcaligenes* sp. to the glassy-winged sharpshooter for use in a paratransgenic control strategy. Curr. Microbiol. 48:327-331
- Blonde, S.E. and Lohner, K. 2000. Combinatorial libraries: a tool to design antimicrobial and antifungal peptide analogues having lytic specificities for structure-activity relationship studies. Biopolymers 55:74-87
- Cammue, B.P.A., De Bolle, M.F.C., Terras, F.R.G., Proost, P., Van Damme, J., Rees, S.B., Vanderleyden, J., Broekaert, W.F. 1992. Isolation and characterization of a novel class of plant antimicrobial peptides from *Mirabilis jalapa* L. seeds. J. Biolog. Chemistry 267:2228-2233
- Casteels, P., Ampe, C., Jacobs, T. and Tempst, P. 1993. Functional and chemical characterization of Hymenoptaecin, an antimicrobial polypeptide that is infection-inducible in the Honeybee (*Apis mellifera*). J. Biolog. Chemistry 268: 7044-7054
- DeGray, G., Rajasekaran, K., Smith, F., Sanford, J. and Daniell, H. 2001. Expression of an antimicrobial peptide via the chloroplast genome to control phytopathogenic bacteria and fungi. Plant Physiology 227:852-862
- Durvasula, R.V., Gumbs, A., Panackal, A., Kruglov, O., Asoy, S., Merrifield, R.D., et al. 1997. Prevention of insect-borne disease: an approach using transgenic symbiotic bacteria. Proc. Natl. Acad. Sci. USA 94:3274-3278
- Jing, W. et al. 2003. The structure of the antimicrobial peptide Ac-RRWWRF-NH<sub>2</sub> bound to micelles and its interactions with phospholipid bilayers. J. Pept. Research 61:219-229.
- Kuzina, L.V., Miller, E.D., Ge, B and Miller, T.A. 2002. Transformation of *Enterobacter gergoviae* isolated from pink bollworm (Lepidoptera: Gelechiidae) gut with *Bacillus thuringiensis* toxin. Curr. Microbiol. 44:1-4
- Maloy, W.L. and Kari, U.P. 1995. Structure-activity studies on magainins and other host defense peptides. Biopolymers 37: 105-122
- Nayler, W.G., Gu, X.H., Casley, D.J. 1989. Sarafotoxin S6c is a relatively weak displacer of specifically bound. Biochem. Biophys. Res. Commun. 161:89-94
- Schroder, J.M. 1999. Epithelial peptide antibiotics. Biochem. Pharmacology 57:121-134
- Selsted, M.E., Novotny, M.J., Morris, W.L., Tang, Y-Q, Smith, W., and Cullor, J.S. 1992. Indolicidin, a novel bactericidal tridecapeptide amide from neutrophils. J. Biol. Chem. 267: 4292-4295
- Silphaduang, U and Noga, E.J. 2001. Peptide antibiotics in mast cells of fish. Nature. 414:268-269

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**DEVELOPMENT OF AN ARTIFICIAL DIET AND EVALUATION OF ARTIFICIAL OVIPOSITIONAL  
SUBSTRATES FOR THE *IN VITRO* REARING OF *GONATOCERUS* SPP. PARASITOIDS  
OF THE EGGS OF THE GLASSY-WINGED SHARPSHOOTER**

**Project Leader:**

Thomas A. Coudron  
USDA, ARS, BCIRL  
Columbia, MO 65203

**Researcher:**

Cynthia L. Goodman  
USDA, ARS, BCIRL  
Columbia, MO 65203

**Collaborators:**

Walker A. Jones  
USDA, ARS, KDLG Subtropical Agric. Res. Center  
Beneficial Insects Research Unit  
Weslaco, TX 78596

Roger Leopold  
USDA, ARS, Red River Valley Agric. Res. Center  
Insect Genetics and Biochemistry Research Unit  
Fargo, ND 58105

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**ABSTRACT**

The intent of this project is to develop an *in vitro* rearing system for one or more of the three mymarid species of *Gonatocerus* currently being reared and released in California to control GWSS. A complete *in vitro* rearing system will include both a growth-enhancing artificial diet for larval and pupal development as well as a suitable oviposition substrate, or “artificial egg”. Initial studies will formulate artificial diets based on those developed previously for hymenopteran parasitoids, with an emphasis being placed on diets for other egg parasitoids. To accomplish this, *Gonatocerus* spp. eggs and/or larvae will be dissected from host eggs and placed in cell culture plates containing selected diets. Comparisons will be made between the development of parasitoids on these artificial diets, and those developing on the natural host. Developmental parameters measured will include extent of development, developmental time per stage, and weight. Once a promising diet is formulated, the reproductive rate and reproductive fitness of adults reared from these diets will be compared by using ovarian scoring and by assessing differences in fecundity and egg viability from crosses of diet-reared and host-reared adult wasps (Wittmeyer et al., 2001; Wittmeyer and Coudron, 2001). Refinement of the diet will be performed by modifying the diet based on its ability to meet the nutritional, phagostimulatory, and endocrine requirements of the parasitoid, and may include the additional of undefined components such as insect or cell-culture derived components. The suitability of artificial eggs, composed of different combinations of membranes and cupule sizes, will be evaluated statistically using pairwise comparisons of the proportion of “artificial eggs” and natural host eggs successfully parasitized by the same number of female *Gonatocerus* parasitoids (SAS, 2002).

**INTRODUCTION**

Surveys of potential biological control agents in Texas (where GWSS is endemic and under natural control) and California revealed that *Gonatocerus* spp. parasitoids are the predominant natural enemy of GWSS in the field, parasitizing between 75-90% of GWSS egg masses (Phillips, 2000; Jones, 2002; Hoddle 2003a). In California, over 90% of the eggs laid by the second generation of GWSS in late summer and early fall are parasitized by *Gonatocerus* spp., however, only 10 – 50% of the eggs laid by the first generation in the early spring are parasitized (Phillips et al., 2004; Hoddle 2003b). This suggests that survival of overwintering adult parasitoids is low, or that the current cohort of species of *Gonatocerus* are not effective in parasitizing GWSS eggs early in the season (Hoddle, 2003b; Jones, pers. comm.). However, augmentation of *Gonatocerus* spp. populations in early spring may be able to significantly reduce the population of GWSS that vector the disease later in the season and could be used to reduce pesticide use thereby aiding in the development of a classical biological control program. The current list of species being considered for biocontrol of GWSS in CA include the solitary egg parasitoids *Gonatocerus ashmeadi* (which accounts for 80-95% observed GWSS egg parasitization in California) and *G. triguttatus* (the primary GWSS egg parasitoid in Texas), as well as the gregarious egg parasitoid *G. fasciatus* (which may have a greater host finding efficiency than the other two) (Hoddle 2003a).

The implementation of current classical and augmentative biological control programs against GWSS has been complicated by a number of factors. Currently, no artificial diet exists for GWSS, and high costs are associated with rearing the sharpshooters in sufficient numbers to provide the necessary quantity of host eggs (Lauziere et al., 2002; Jones, pers. comm.). Long-term stockpiling of host eggs is not feasible at this time because host acceptance declines after refrigeration for 20 days at 13°C, and parasitized eggs only remain viable for 7 days at 2°C (Leopold, 2003). Consequently, augmentation of *Gonatocerus* spp. in many areas of California relies on the labor-intensive process of rearing the parasitoid on host eggs collected from the field (Jones, pers. comm.). Thus, the development of an artificial diet and ovipositional substrate as part of an *in vitro* mass rearing system for *Gonatocerus* spp. has a number of potential advantages over current rearing techniques. Additionally, *in vitro* rearing would also be more easily automated, reducing labor costs (Li-Ying, 1992; Qin, Beijing Univ., pers. comm.) and would provide an easier means for studying the reproductive and nutritional physiology of *Gonatocerus* spp.

Efforts to develop an artificial diet capable of supporting larval and pupal development will initially focus on testing established diets formulated for the *in vitro* rearing of other egg parasitoids, e.g., those used for rearing lepidopteran egg parasitoids including several *Trichogramma* spp. (Hoffman et al., 1975; Li-Ying 1992; Consoli and Parra, 1997; Xie et al., 1997; Grenier et al., 1998; Qin, Beijing Univ. pers. comm.), *Telenomus heliothidis* (Strand et al., 1988), and *Ooencyrtus* spp. (Masutti et al., 1994; Lee and Lee, 1994); a coleopteran egg parasitoid, *Edovum puttleri* (Hu et al., 1999; Hu et al., 2001), and a pentatomid egg parasitoid, *Trissolcus basal* (Volkoff et al., 1992). For studies on the development of an artificial ovipositional substrate, membranes that will be derived from a variety of sources will be tested, such as: oxygen-permeable films used for mass rearing *Trichogramma* spp. (Qin, Beijing University, pers. comm.), parafilm (Wittmeyer et al., 2001; Cooperband and Vinson, 2001), and polycarbonate, polyvinylchloride, polyethylene, and/or polypropylene membranes (Masutti et al., 1994; Morrison et al., 1983; Consoli and Parra 1999).

## OBJECTIVES

1. Formulate an artificial diet capable of supporting the development and reproduction of *Gonatocerus* spp. parasitoids of the eggs of glassy-winged sharpshooter, *Homalodisca coagulata*.
2. Screen, modify, and evaluate existing materials for their suitability as ovipositional substrates for these egg parasitoids.
3. Develop and optimize an *in vitro* rearing unit, consisting of an artificial diet and ovipositional substrate, that can be utilized for *Gonatocerus* spp. oviposition, parasitoid development, and release.

## RESULTS AND CONCLUSIONS

This project has just been funded. Preparation of quarantine facilities is complete and the identification of insect cultures to be used in our studies is underway. The process to hire an additional researcher has been initiated. Preliminary experiments have been conducted in collaboration with Leopold at ARS in Fargo that indicate cold-storage processes should offer suitable method(s) to preserve the natural host of the parasitoid for these studies.

## REFERENCES

- Consoli FL, Parra JRP, 1997. Biological Control 8: 172-176.
- Cooperband MF, Vinson SB, 2001. Biol. Control 17(1): 23-28.
- Grenier S, Han SC, Chapelle L, Liu WH, Guillaud J, 1998. Biocontrol Science & Technology. 8(4): 589-596.
- Hoffman JD, Ignoffo CM, Dickerson WA, 1975. Ann. Entomol. Soc. Am. 68: 335-336.
- Hoddle M, 2003a. In: Proc. Pierce's Dis. Res. Symp. San Diego, CA. CDFA.
- Hoddle M, 2003b. In: Proc. Pierce's Dis. Res. Symp. San Diego, CA. CDFA.
- Hu JS, Gelman DB, Bell RA, 2001. BioControl 46: 43-60.
- Hu JS, Gelman DB, Bell RA, Lynn DE, 1999. Arch. Insect Biochem. Physiol. 40: 173-182.
- Jones WA, 2002. In: Proc. Pierce's Dis. Res. Symp. San Diego, CA. CDFA.
- Lauziere I, Ciomperlik M, Wendell L, 2002. In: Proc. Pierce's Dis. Res. Symp. San Diego, CA. CDFA.
- Lee HP, and Lee KS, 1994. Korean J. of Entomol. 24: 311-316.
- Leopold RA, 2003. In: Proc. Pierce's Dis. Res. Symp. San Diego, CA. CDFA.
- Li-Ying L, 1992. Korean J. Applied Entomol. 31: 241-246.
- Masutti L, Battisti A, Milani N, Zanata M, Zanazzo G, 1994. Entomophaga 38: 327-333.
- Morrison RK, Nettles WC, Jr., Ball D, Vinson SB. 1983. Southwestern Entomol. 8: 248-251.
- Phillips PA, Wilen CA, Varela LG, 2004. URL: [www.axp.ipm.ucdavis.edu/PMG/PESTNOTES/pn7492.html](http://www.axp.ipm.ucdavis.edu/PMG/PESTNOTES/pn7492.html)
- Phillips PA, 2000. KAC Plant Protect Quarterly 10: 6-7.
- Strand MR, Vinson SB, Nettles WC, Jr., Xie ZN, 1988. Entomologia Experimentalis et Applicata 46: 71-78.
- Volkoff N, Vinson SB, Wu ZX, Nettles WC Jr., 1992. Entomophaga 37: 141-148.
- Wittmeyer JL, Coudron TA, Adams TS, 2001. Invertebrate Reproduction & Development 39: 9-20.
- Wittmeyer JL, Coudron TA, 2001. J. Econ. Entomol. 94: 1344-1352.
- Xie ZN, Wu ZX, Nettles WC Jr., Saldana G, Nordlund DA, 1997. Biological Control 8: 107-110.

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Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

## DESIGN OF CHIMERIC ANTI-MICROBIAL PROTEINS FOR RAPID CLEARANCE OF *XYLELLA*

### Project Leaders:

Abhaya M. Dandekar  
Dept. of Plant Science  
University of California  
Davis, CA 95616

Goutam Gupta  
B-1, MS M888, LANL  
Los Alamos, NM 87545

Elizabeth Hong-Geller  
B-1, MS M888, LANL  
Los Alamos, NM 87545

Karen McDonald  
Chem. Engr. and Material Sci.  
University of California  
Davis, CA 95616

### Collaborators:

George Bruening  
Dept. of Plant Pathology  
University of California  
Davis, CA 95616

Edwin L. Civerolo  
SJVASC, MS  
Parlier, CA 93468

Patrick R. Shiflett  
M888, B Division, LANL Los  
Alamos, NM 87545

Pat J. Unkefer  
B-3, MS E529, LANL  
Los Alamos, NM 87545

Cliff J. Unkefer  
B-3, MS G758, LANL  
Los Alamos, NM 87545

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### ABSTRACT

*Xylella fastidiosa* (*Xf*), is a gram-negative xylem-limited bacterium and causative agent of Pierce's disease (PD) in California grapevines. During very early stages of *Xf* infection, specific carbohydrates/lipids/proteins on the outer membrane of *Xf* interact with plant cells and are important for virulence (3). Design of a protein inhibitor that interrupts this step of the plant-*Xf* interaction will be useful in anti-microbial therapy and controlling PD. Traditionally, antibiotics are prescribed as a preferred therapy; however, a pathogen often develops antibiotic resistance and escapes their anti-microbial action (4). In this UC/LANL project, we propose a novel protein-based therapy that circumvents the shortcomings of an antibiotic. We have designed a chimeric anti-microbial protein with two functional domains. One domain (called the surface recognition domain or SRD) will specifically target the bacterium outer-membrane whereas the other will lyse the membrane and kill *Xf*. In this chimera, Elastase is the SRD that recognizes mopB, the newly discovered *Xf* outer membrane protein (5). The second domain is Cecropin B, a lytic peptide that targets and lyses gram-negative bacteria. We have successfully tested each of these components individually and demonstrated that they each (Elastase and Cecropin B) display activity against *Xf*, which is increased when both proteins are combined. We have tested Elastase against purified mopB and intact *Xf* cells and found that mopB is degraded in both cases, suggesting that it is potentially a target for Elastase. The HNE-GSTA-Cecropin B chimera gene has been synthesized and is currently being cloned into vectors for overexpression in insect and grapevine cells in order to test its activity against *Xf in vitro*. We have also initiated transgenic grapevine cultures expressing a pear polygalacturonase inhibiting protein that is secreted into the medium using a CELLline 350 bioreactor. In the future, we plan to use this system to test secretion and anti-*Xf* of the chimeric protein.

### INTRODUCTION

Globally, one-fifth of potential crop yields are lost due to plant diseases primarily of bacterial origin. *Xylella fastidiosa* (*Xf*) is a devastating bacterial pathogen that causes PD in grapevines, citrus variegated chlorosis (CVC) in citrus, and leaf scorch disease in numerous other agriculturally significant plants including almonds in California (<http://danr.ucop.edu/news/speeches>). Since the glassy-winged sharpshooter (an insect vector) efficiently transmits PD, a great deal of effort has been focused on using insecticides to localize and eliminate the spread of this disease. However, the availability of the whole genome sequences of PD and CVC strains of *Xf* offer new avenues to directly target and inactivate the pathogen. In this project, we propose a structure-based approach to develop chimeric anti-microbial proteins for rapid destruction of *Xf*. The strategy is based upon the fundamental principle of innate immunity that plants recognize and clear pathogens in rapid manner (1-2). Pathogen clearance by innate immunity occurs in three sequential steps: pathogen recognition, activation of anti-microbial processes, and finally pathogen destruction by anti-microbial processes. Different sets of plant factors are involved in different steps of innate immunity. Our strategy of combining a pathogen recognition element and a pathogen killing element in the chimeric molecule is a novel concept and has several short and long term impacts.

## OBJECTIVES

- Objective 1:
- a) Utilize literature data and computer modeling to identify an SRD that specifically targets mopB (Elastase)
  - b) Utilize literature data and computer modeling to identify a useful Cecropin (i.e., Cecropin B)
  - c) *In vitro* testing of anti-*Xylella* activity of the mopB-specific SRD (Elastase) and *Xylella*-specific Cecropin B and demonstration of synergistic killing effect due to the combined use of Elastase and Cecropin B.
- Objective 2:
- a) Design and construction of synthetic gene encoding Elastase-Linker-Cecropin B Chimeric protein
  - b) Expression Elastase-Linker-Cecropin B in insect and plant cells and testing activity *in vitro*.
- Objective 3:
- a) Expression in transgenic plants
  - b) Testing for anti-*Xylella* activity *in planta* and testing for graft transmissibility.

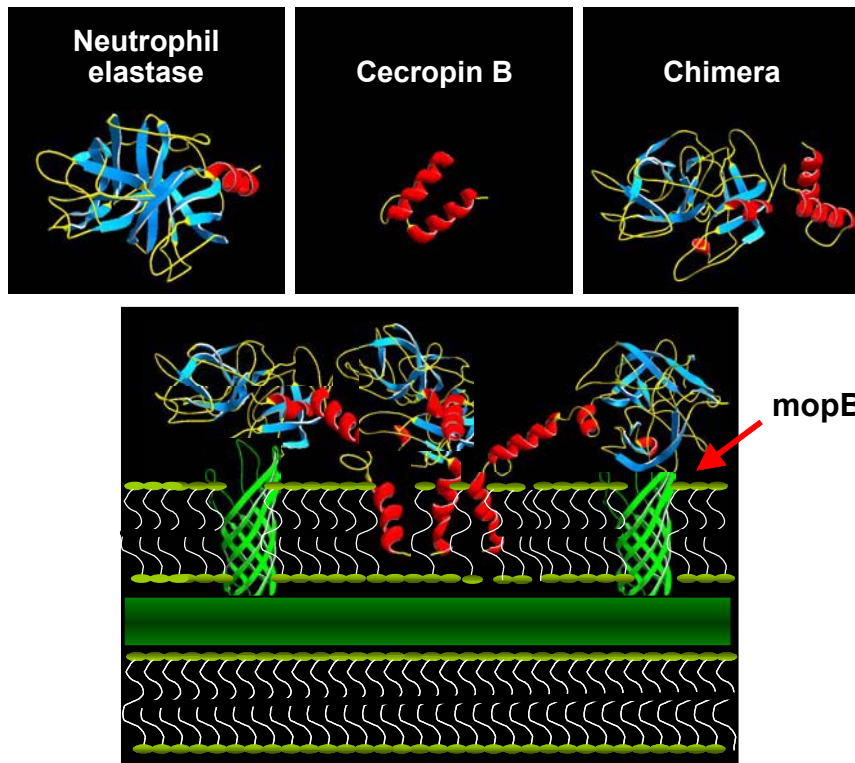
## RESULTS AND CONCLUSION

Human Neutrophil Elastase (HNE) (6) was chosen as our first SRD. Neutrophils contain a variety of proteins that enable the cells to migrate toward and eliminate microbial pathogens (7). Until 1991, no specific antibacterial activity had been ascribed to HNE (8). However recent research has established that HNE is the only human neutrophil protein, which is capable of individually killing *Borrelia burgdorferi*, the causative agent of Lyme disease (9, 10). Furthermore, it is known that HNE can augment the cidal properties of other active proteins (11). Sequence-structure analysis of mopB revealed that it contained an specific cleavage site for HNE that is exposed on the surface. We have studied the efficacy of HNE in combination with the antibacterial peptide Cecropin B, that inserts preferentially into the lipid bilayer of gram-negative bacteria, in killing *Xf*. Measuring the number of colony forming units remaining after the bacterium was exposed to HNE, Cecropin B and the combination of both, we found that HNE greatly stimulates the lysis induced by Cecropin B. In addition, we found that Mop B was partially digested by HNE after incubating either purified Mop B or *Xf* cells with HNE for an hour. Based on these preliminary results, we have designed a chimeric protein of Cecropin B and HNE; in order to stabilize the Cecropin B peptide and enhance the overall affinity of the ligands for the bacterial surface. The covalent attachment of Cecropin B to HNE is proposed to increase the stability of the peptide by lowering the conformational entropy of its unfolded state and to increase the overall affinity for the bacterial surface by minimizing the degrees of motion at the binding site, thereby increasing binding between the ligands and the surface.

Our strategy began with the generation of a 3-D model of the chimera. The modeling was based on published protein data bank (PDB) structures of HNE and nuclear magnetic resonance structures of peptides homologous to Cecropin B. A short G-S-T-A peptide linker was inserted between the C-terminus of HNE and the N-terminus of Cecropin B to allow both functional domains to make contact with the bacterial surface simultaneously without steric interference. Energy minimization and molecular dynamics analysis using the AMBER 7.0 force field indicated that the chimera forms a stable structure. The HNE-GSTA-Cecropin B chimera gene was synthesized and is currently being cloned into a baculovirus vector for overexpression in insect cells. The chimera will be purified from insect cells and tested for its activity against *Xf in vitro*. The chimera will be also cloned into a plant vector for transformation of grape embryogenic callus growing in a CELLline 350 bioreactor where they will be analyzed for the production and anti-*Xf* activity of the secreted protein. We will choose the most promising embryogenic lines for plant regeneration. The plant expression vector will have necessary regulatory sequences to facilitate transcription and extracellular delivery of the protein product. Currently we are investigating grapevine embryogenic callus for the extracellular production of the pear polygalacturonase inhibiting protein (pPGIP). This protein has been found in the xylem exudate of transgenic grapes expressing the pPGIP gene and will be used to modify delivery of the chimeric protein to grapevine xylem tissues.

IVGRRARPHAWPFMVSLQLRGGHFCGATLIAPNFVMSAAHCVANVNVRAVRVVLGAHNLSRREPTR  
QVFAVQRIFEDGYDPVNLLNDIVLQLNGSATINANVQVAQLPAQGRRLGNGVQCLAMGWLLGRNRG  
IASVLQELNVTVTSLCRRSNVCTLVRGRQAGVCFGDSGSPLVCNGLIHGIAFVRGGCASGLYPDAFAP  
VAQFVNWIDSIIQGSTAKWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL

**Figure 1. HNE-cecropin B chimeric amino acid sequence.** HNE is attached to cecropin B (shown in bold) by the GSTA linker, which is underlined.



**Figure 2. Design and mechanism of chimeric protein targeted to *X. fastidiosa*.** The top panel shows the two domains of the chimera in separate planes: neutrophil elastase (1HNE from PDB) is on the left. A homology model of cecropin B is shown in the middle. The right plane shows the energy minimized model of the elastase-cecropin B chimera. The bottom panel is a schematic of the hypothetical mechanism of the chimeric protein. Elastase binds to and cleaves a specific loop on the *X. fastidiosa* outer membrane protein mopB. This action brings cecropin B in close contact with the membrane, where it associates with other cecropin molecules and disrupts the membrane by forming a pore, thereby disabling the bacterium.

## REFERENCES

1. Cohn, J., Sessa, G., and Martin, G.B. (2001). Innate immunity in plants. *Curr. Opin. Immunol.* 13:55-62.
2. Magor, B.G. and Magor, K.E. (2001). Evolution of effectors and receptor of innate immunity. *Dev. Comp. Immunol.* 25:651-682.
3. Pieters, J. (2001). Evasion of host cell defense by pathogenic bacteria. *Curr. Opin. Immunol.* 13:37-44.
4. Baquero, F. and Blazquez, J. (1997). Evolution of antibiotic resistance. *Trends Ecol. Evol.* 12:482-487.
5. Bruening, G., Civerelo, E., Kirkpatrick, B., and Gilchrist, D. (2002). Virulence analysis of The Pierce's Disease Agent *X. fastidiosa*. PD research symposium Dec 15-18 2002, San Diego, CA.
6. Sinha, S., W. Watorek, et al. (1987). Primary structure of human neutrophil Elastase. *Proc. Natl. Acad. Sci. USA* 84: 2228-2232.
7. Elsbach, P., and Weiss, J. (1988). Phagocytic cells: oxygen-independent anti-microbial systems. *In* *Inflammation: basic principles and clinical correlates*. Gallin, J.L., Goldstein, I.M. and Snuderman, R. (Ed.). Raven Press, New York, USA, pp 445-470.
8. Wasiluk, K. R., K. M. Skubitz, et al. (1991). Comparison of granule proteins from human polymorphonuclear leukocytes which are bactericidal toward *Pseudomonas aeruginosa*. *Infection and Immunity* 59: 4193-4200.
9. Garcia, R., L. Gusmani, et al. (1998). Elastase is the only human neutrophil granule protein that alone is responsible for in vitro killing of *Borrelia burgdorferi*. *Infection and Immunity* 66(4): 1408-1412.
10. Lusitani, D., S. E. Malawista, et al. (2002). *Borrelia burgdorferi* are susceptible to killing by a variety of human polymorphonuclear leukocyte components. *The Journal of Infectious Diseases* 185: 797-804.
11. Miyasaki, K. T. and Bodeau, A.L. (1991). In vitro killing of *Actinobacillus actinomycetemcomitans* and *Capnocytophaga* spp. by human neutrophil cathepsin G and Elastase. *Infection and Immunity* 59: 3015-3020.

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**EXTENSIVE SEQUENCE DIVERGENCE IN THE ITS2 RDNA FRAGMENT IN A POPULATION  
OF *GONATOCERUS ASHMEADI* FROM FLORIDA: PHYLOGENETIC RELATIONSHIPS  
OF *GONATOCERUS* SPECIES**

**Project Leader:**

Jesse H. de León  
USDA, ARS  
BIRU  
Weslaco, TX 78596

**Cooperators:**

Walker A. Jones  
USDA, ARS  
BIRU  
Weslaco, TX 78596

David J. W. Morgan  
CDFA  
Mount Rubidoux Field Station  
Riverside, CA 92501

Russell F. Mizell, III  
University of Florida  
NREC-Monticello  
Quincy, FL 32351

**Reporting period:** The results reported here are from work conducted from fiscal year 2003 to fiscal year 2004.

**ABSTRACT**

The aim of the present study was to resolve the genetic relationships of geographic populations of *Gonatocerus ashmeadi*, a primary egg parasitoid of the glassy-winged sharpshooter. A phylogenetic approach was implemented by sequencing the Internal Transcribed Spacer-2 (ITS2) region of several individuals per population. In addition, the phylogenetic relationships of several *Gonatocerus* species were also determined. Six geographic populations of *G. ashmeadi* were analyzed: Quincy, FL (QFL), two populations from Weslaco, TX (WTXa and WTXb), Louisiana (LA), San Antonio, TX (SATX), and California (CA). The percentage divergence (%D) of the ITS2 sequences, as measured by genetic distance, was small among LA, SATX, and CA (0.10-1.10%); whereas, the %D for QFL vs these populations was extremely high (65.9-69.8%). A Neighbor-Joining distance tree separated the QFL population into a separate clade supported by very high bootstrap values (100%). When the Weslaco populations were included in the analysis, they clustered into two distinctive clades, WTXb clustered with QFL and WTXa clustered with the rest of the populations; again very high bootstrap values (100%) supported the topology of the distance tree. These results indicate the presence of sympatric strains in Weslaco. The phylogenetic analysis of several *Gonatocerus* species clustered the respective species into North and South American clades. The %D of the QFL population fell within the range (75.4-87.2%) of the South American *Gonatocerus* species and clustered within the South American clade. The present molecular phylogenetics results provide strong evidence that *G. ashmeadi* from Florida may be a different species. In addition, the data is suggestive that the origin of *G. ashmeadi* in California is the Texas region, including the closely located Louisiana. The findings of the present study are important to the Glassy-winged Sharpshooter/Pierce's Disease biological control program in California.

**INTRODUCTION**

*Gonatocerus ashmeadi* (Girault) (Hymenoptera: Mymaridae) is a primary egg parasitoid of *Homalodisca coagulata* (Say) (Homoptera: Cicadellidae), the Glassy-winged Sharpshooter (Huber 1998). A biological control program is currently in progress in California against *H. coagulata* because this xylem feeding leafhopper is a serious economic pest that vectors a strain of *Xylella fastidiosa* (Wells), a bacterium that causes Pierce's Disease in grapevines. Accurate identification of natural enemies is critical to the success of classical biological control programs. Lack of proper identification procedures has affected the early stages of several projects (Messing and Aliniaze 1988; Löhr *et al.* 1990). The Internal Transcribed Spacer regions (ITS-1 and -2) have been used extensively to examine the taxonomic status of species and for diagnostic purposes, and success with this approach has been reviewed by Collins and Paskewitz (1996).

**OBJECTIVES**

1. Determine the phylogenetic relationships of geographic populations of *G. ashmeadi*.
2. Determine the phylogenetic relationships of several *Gonatocerus* species, including candidate species from South America (Argentina).

**RESULTS AND CONCLUSIONS**

***Genetic Relatedness Among Geographic Populations Of G. ashmeadi***

Levels of genetic divergence in the ITS2 rDNA fragment among populations were determined by calculating the pairwise estimates for genetic distance (Table 1). Recently, we determined by ISSR-PCR DNA fingerprinting that *G. ashmeadi* geographic populations were highly differentiated (de León and Jones 2004). The data demonstrated that the Quincy, FL (QFL) population had the highest gene diversity value. In addition, the data indicated that two Weslaco, TX populations collected at different times of the year were divergent or differentiated from each other and gave a first clue as to the presence of sympatric strains in Weslaco. As seen on Table 1, the sequence percentage divergence (%D) between the QFL population and the rest of the *G. ashmeadi* geographic populations (LA, SATX, WTXa, and CA) was extremely high, ranging from 65.9 to 69.8%. The %D between QFL and the outgroup population (*G. morrilli*) ranged from 77.8-81.2%, whereas LA, SATX, WTXa, and CA ranged from 31.4 to 37.0% compared to the outgroup. The %D among LA, SATX, WTXa, and CA populations was extremely low, 0.10 to 1.10%, indicating the very close genetic similarity among these geographic populations. This range is within the intra-population variation found within each of these populations. A phylogenetic analysis (Fig. 1A) demonstrated that the QFL and the LA, SATX, WTXa, and CA populations formed two distinct clades supported by extremely high bootstrap support values; in most cases they were at 100%. Our second goal was to confirm whether sympatric strains of *G. ashmeadi* indeed existed in Weslaco. Table 1 shows that the %D between QFL

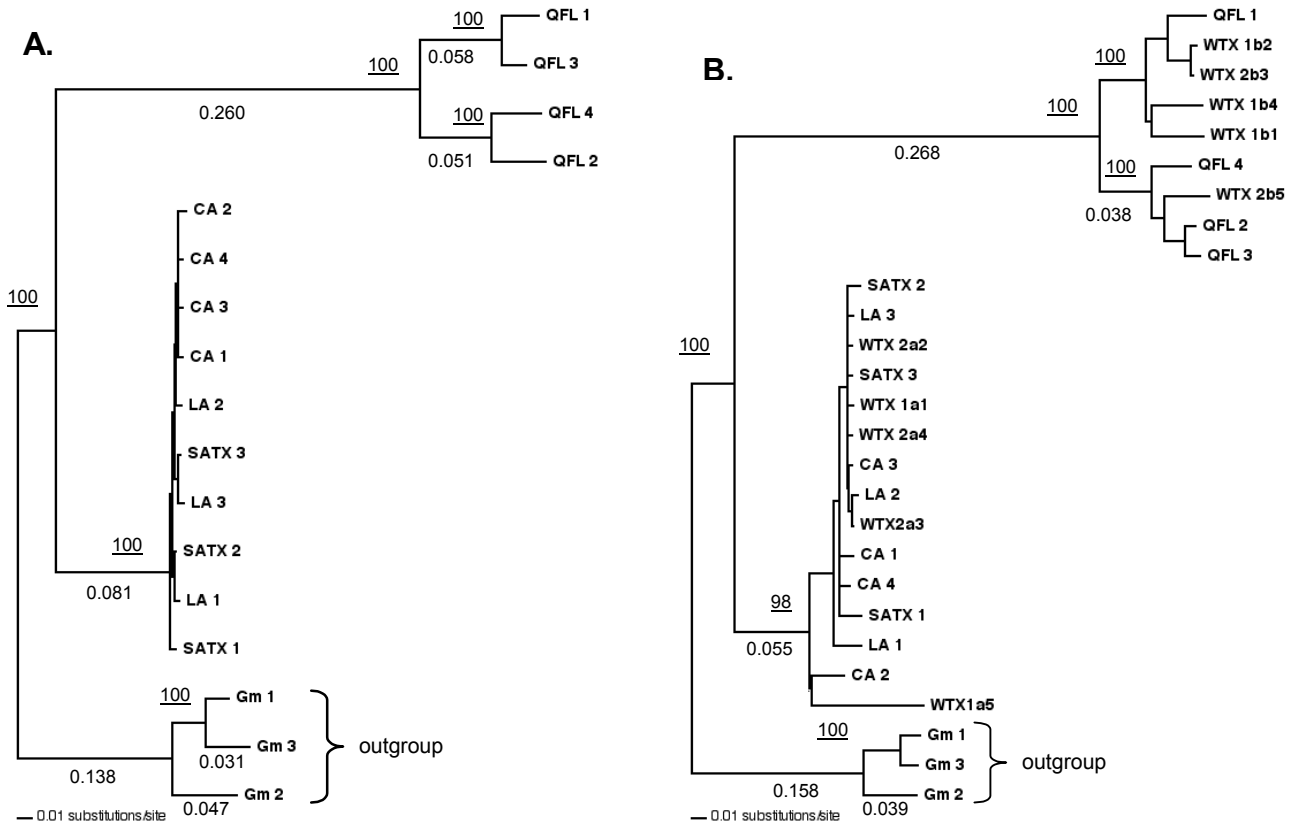
and WTXb is very low (0.00-0.40%) and falls within the range of the intra-populational variation. In contrast, the %D between WTXb and the rest of the populations falls within the same range that the QFL population (65.9-69.8%) fell in. The phylogenetic analysis of all populations (Fig. 1B), including the two Weslaco populations (WTXa and WTXb) demonstrated that these two populations fell on separate clades, confirming the existence of sympatric strains in Weslaco. WTXb clustered with QFL and WTXa clustered with the rest of the *G. ashmeadi* populations. Again, the distance tree is supported by extremely high bootstrap support values (100%). The very high %D values indicate that the QFL and WTXb complex diverged some time ago. The earliest record of *G. ashmeadi* in California was from 1979 (Vickerman et al. 2004) and recently, we showed that a subset of glassy-winged sharpshooters in California had their origin in central Texas (de León et al. 2004). The present results lend support to the idea that *G. ashmeadi* may have its origins in central Texas (SATX) (including the very closely located Louisiana). So it is possible that *G. ashmeadi* was transported to California along with the Glassy-winged Sharpshooter from central Texas.

#### Phylogenetic Relationships Among *Gonatocerus* Species

Resolution of relationships requires information about variability not only at the level of populations within a species but also between species (Narang et al. 1993; Unruh and Woolley 1999); therefore, a molecular systematic approach was undertaken with various *Gonatocerus* species, including candidates from South America (Argentina). For the pairwise sequence distance analyses, the *G. ashmeadi* populations (LA, SATX, WTXa, and CA) that formed one clade in fig. 1 were pooled (Ga\*, Table 2) and compared to the rest of the *Gonatocerus* species. The %D values among these populations were very low (0.10-0.90%), falling within the range of the intra-specific variation seen within each individual species. The %D of *G. trigtutatus* (Gt) and *G. morrilli* (Gm) vs Ga\* is 15.8-17.9 and 35.0-38.9%, respectively. In contrast, the %D of *G. ashmeadi* from Florida [Ga(FL)] vs Ga\* is 75.4-79.8%, these values fall within the %D range of all South American species (Table 2). This is demonstrated visually on the phenogram in Fig. 2 with very strong bootstrap values supporting the topology of the Nieghbor-Joining distance tree. As seen from the phenogram, the North and South American *Gonatocerus* species are separated into their perspective clades. It is interesting to note that Ga(FL) is more closely related to *G. metanotalis* (Gmet) (8.30-9.00%), a South American species than it is to any North America species (Fig. 2). The *Gonatocerus* species more closely related to Ga\* is Gt (15.8-17.9%). The present results showing extensive sequence divergence at the ITS2 rDNA fragment in a population of *G. ashmeadi* from Florida lends strong support to the fact that these individuals may actually be another species or rather *G. ashmeadi* exists in nature as a species-complex. Our results are in contrast with those of Vickerman et al. (2004). In our studies we performed a phylogenetic analyses of the ITS2 rDNA sequences. In addition, Vickerman et al. (2004) demonstrated that populations of *G. ashmeadi* from Florida vs other geographic regions were able to hybridize. We have not yet performed these types of studies, but it may be necessary to extend these crossing studies to the F2 generation to seen a negative effect or as demonstrated by Wu et al. (2004) a negative effect was not seen until backcrosses were performed. The findings of the present study are important to the Glassy-winged Sharpshooter/Pierce's Disease biological control program in California.

**Table 1. Pairwise sequence distances (range) of ITS-2 rDNA fragments from geographic populations of *G. ashmeadi* showing percentage divergence.** The alignment program ClutstalW (Thomas *et al.* 1994) from DNASTar was utilized for this analysis. To account for intra- and inter-populational variation, several individuals (3-4) were included. QFL, Quincy, Florida; WTXb, Weslaco, TX; LA, Louisiana; SATX, San Antonio, TX; WTXa, Weslaco, TX; CA, California; Gm, *G. morrilli* (outgroup). Relate to figure 1B.

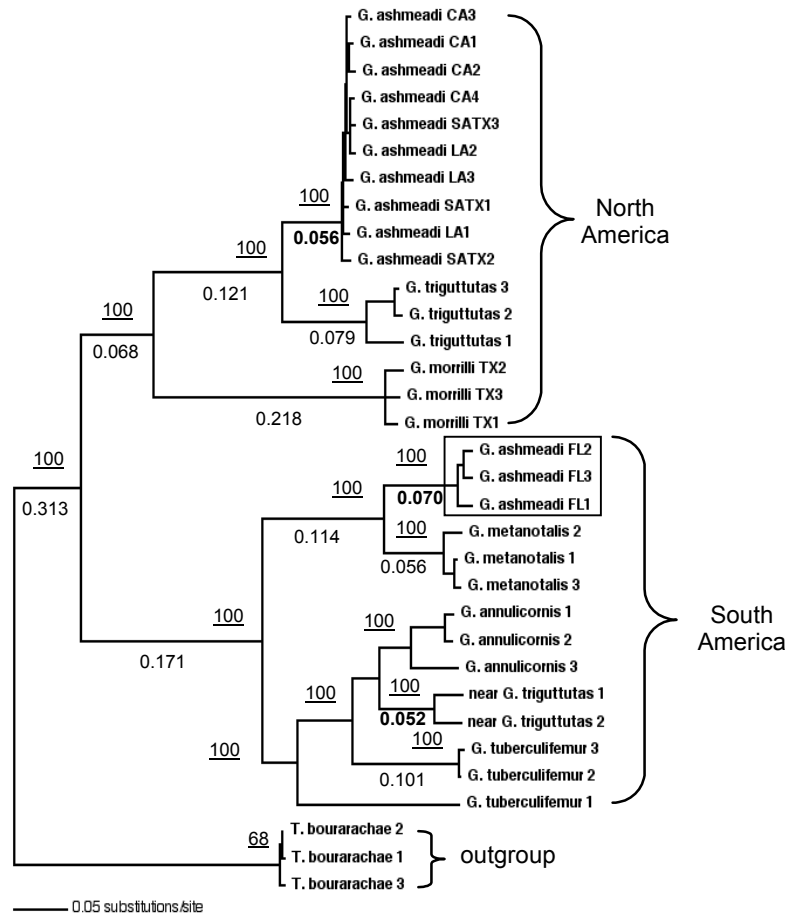
Pop	QFL	WTXb	LA	SATX	WTXa	CA	Gm
QFL	0.10-0.40						
WTXb	0.00-0.40	0.00-0.10					
LA	68.0-69.8	68.1-70.4	0.60-0.90				
SATX	68.2-69.8	67.9-70.8	0.30-0.80	0.20-0.90			
WTXa	67.1-69.5	66.6-70.1	0.20-0.70	0.20-0.90	0.10-0.90		
CA	65.9-67.6	66.0-67.9	0.80-1.00	0.60-1.10	0.30-1.00	0.20-0.80	
Gm	77.8-81.2	77.6-82.3	32.3-36.3	31.4-37.0	31.8-40.6	36.3-36.7	0.00-0.30



**Figure 1.** Phenograms of ITS2 rDNA sequence fragments from geographic populations of *G. ashmeadi*. Analyses were performed with the alignment program ClustalX (Thompson *et al.* 1997) and the Neighbor-Joining trees were created with the phylogenetic program PAUP 4.0 (Swofford 2002). In the genetic distance trees *G. morrilli* are included as an outgroup, displaying branch lengths (below branches) and bootstrap values (above branches underlined), as percentage of 1000 replications. Trees are presented both without Weslaco, TX populations (**A**) and with Weslaco, TX populations (**B**). To account for intra- and inter-populational variation, several randomly chosen individuals (3-4) were included.

**Table 2.** Pairwise sequence distances (range) of ITS-2 rDNA fragments from *Gonatocerus* species showing percentage divergence. The alignment program ClustalW (Thomas *et al.* 1994) from DNASTar was utilized for this analysis. To account for intra- and inter-specific variation, several individuals (2-3) were included. Ga\*, *G. ashmeadi* (California, San Antonio, TX, and Louisiana were pooled for a total of 10 individuals); Gt, *G. triguttatus* (TX); Gm, *G. morrilli* (TX); and candidate South American (Argentina) species: Gann, *G. annulicornis*; nGt, near *G. triguttatus*; Gtub, *G. tuberculifer*; Ga(FL), *G. ashmeadi* (Quincy, FL USA); Gmet, *G. metanotalis*; and Tb, *Trichogramma bourarachae* (outgroup).

<i>G species</i>	<i>Ga*</i>	<i>Gt</i>	<i>Gm</i>	<i>Gann</i>	<i>nGt</i>	<i>Gtub</i>	<i>Ga(FL)</i>	<i>Gmet</i>	<i>Tb</i>
Ga*	0.10-0.90								
Gt	15.8-17.9	0.10-0.20							
Gm	35.0-38.9	41.7-45.5	1.80-1.80						
Gann	82.4-87.2	97.5-101	87.0-88.1	0.00-0.10					
nGt	80.0-83.5	94.8-97.3	82.7-84.2	3.40-3.60	0.10-0.10				
Gtub	78.0-82.0	90.8-92.0	81.4-84.1	11.5-12.1	11.6-11.8	0.10-0.50			
Ga(FL)	75.4-79.8	88.4-90.2	84.3-87.0	37.7-39.3	36.7-38.1	35.9-36.4	0.10-1.00		
Gmet	76.2-80.4	87.6-89.4	85.5-88.2	35.4-36.4	34.7-35.3	34.9-36.1	8.30-9.00	0.10-0.40	
Tb	84.8-92.5	87.2-91.5	88.4-90.4	66.1-67.6	69.0-70.3	68.5-70.5	77.3-79.6	74.2-76.2	0.20-0.90



**Figure 2.** Phenograms of ITS2 rDNA sequence fragments from *Gonatocerus* egg parasitoid species, including candidate species from South America (Argentina). Analysis was performed with the alignment program ClustalX (Thompson *et al.* 1997) and the Nieghbor-Joining trees were created with the phylogenetic program PAUP 4.0 (Swofford 2002). In the genetic distance trees *Trichogramma bourarachae* (1, AF043624; 2, AF043625; 3, AF043626) are included as an outgroup, displaying branch lengths (below branches) and bootstrap values (above branches underlined), as percentage of 1000 replications. To account for intra- and inter-specific variation, several randomly chosen individuals (2-4) were included.

## REFERENCES

- de León JH, Jones WA. 2004. Genetic differentiation among geographic populations of *Gonatocerus ashmeadi* (Hymenoptera: Mymaridae), the predominant egg parasitoid of *Homalodisca coagulata* (Homoptera: Cicadellidae). *Journal of Insect Science* (in press).
- de León JH, Jones WA, Morgan DJW. 2004. Population genetic structure of *Homalodisca coagulata* (Homoptera: Cicadellidae), the vector of the bacterium *Xylella fastidiosa* causing Pierce's disease in grapevines. *Annals of the Entomological Society of America* 97: 809-818.
- Huber JT. 1998. The species groups of *Gonatocerus* Nees in North America with a revision of the *sulphuripes* and *ater* groups (Hymenoptera: Mymaridae). *Memoirs of the Entomological Society of Canada* 141: 1-109.
- Löhr BA, Varela M, Santos B. 1990. Exploration for natural enemies of the cassava mealybug, *Phenococcus manihoti* (Homoptera: Pseudococcidae), in South America for the biological control of this introduced pest in Africa. *Bulletin of Entomological Research* 80: 417-425.
- Messing RH, Aliniaze MT. 1988. Hybridization and host suitability of two biotypes of *Trioxys pallidus* (Hymenoptera: Aphididae). *Annals of the Entomological Society of America* 81: 6-9.
- Narang SK, Tabachnick WJ, Faust RM. 1993. Complexities of population genetic structure and implications for biological control programs. In: Narang SK, Barlett AC, Faust RM, editors. *Applications of Genetics to Arthropods of Biological Control Significance*, 19-52. CRC Press Inc, Boca Raton, Florida.
- Porter CH, Collins FH. 1991. Species-diagnostic difference in a ribosomal DNA internal transcribed spacer from the sibling species *Anopheles freeborni* and *Anopheles hermsi* (Diptera: Culicidae). *American Journal of Tropical Medicine and Hygiene* 45, 271-279.
- Thompson JD, Gibson TJ, Plewniak F, Higgins DG. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 24: 4876-4882.

- Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22:4673-4680.
- Swofford DL. 2002. PAUP\*. Phylogenetic Analysis Using Parsimony (\*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.
- Vickerman DB, Hoddle MS, Triapitsyn S, Stouthamer R. 2004. Species identity of geographically distinct populations of the glassy-winged sharpshooter parasitoid *Gonatocerus ashmeadi*: morphology, DNA sequences, and reproductive compatibility. *Biological Control* 31: 338-345.
- Unruh TR, Woolley JB. 1999. Molecular Methods in Classical Biological Control. In: Van Driesche RG, Bellows TS, Jr., editors. *Biological Control*, 57-85. Chapman and Hall, NY.
- Wu Z, Hopper KR, O'Neil RJ, Voegtlin DJ, Prokrym DR, Heimpel GE. 2004. Reproductive compatibility and genetic variation between two strains of *Aphelinus albipodus* (Hymenoptera: Aphelinidae), a parasitoid of the soybean aphid, *Aphis glycines* (Homoptera: Aphididae). *Biological Control* 31: 331-319.

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# GENETIC DIFFERENTIATION AMONG GEOGRAPHIC POPULATIONS OF *GONATOCERUS ASHMEADI*, A PRIMARY EGG PARASITOID OF THE GLASSY-WINGED SHARPSHOOTER

## Project Leader:

Jesse H. de León  
USDA, ARS  
BIRU  
Weslaco, TX 78596

## Cooperators:

Walker A. Jones  
USDA, ARS  
BIRU  
Weslaco, TX 78596

David J. W. Morgan  
CDFA  
Mount Rubidoux Field Station  
Riverside, CA 92501

Russell F. Mizell, III  
University of Florida  
Quincy, FL 32351

**Reporting period:** The results reported here are from work conducted from fiscal year 2003 to fiscal year 2004.

## ABSTRACT

The aim of genetically comparing different populations of the same species of natural enemies is to identify the strain that is most adapted to the environment where it will be released. In the present study, Inter-Simple Sequence Repeat-Polymerase Chain Reaction (ISSR-PCR) was utilized to estimate the population genetic structure of *Gonatocerus ashmeadi*. Six populations from throughout the U. S. and a population from Argentina identified as near *G. ashmeadi* were analyzed. Four populations [California (CA), San Antonio, TX (SATX), Weslaco, TX (WTX-2), and Quincy, Florida (QFL)] were field collected and two [Louisiana (LA) and Weslaco, TX (WTX-1)] were reared. Three ISSR-PCR reactions were pooled to generate 41 polymorphic markers among the six U. S. populations. Nei's expected heterozygosity values ( $h$ ), including the reared population from Louisiana were high (9.0-14.3%) for all populations, except for a reared population from WTX-1 (2.9%). The total genetic diversity value ( $H_t$ ) for the field populations was high (23%). Interestingly, the Florida population that was collected from one egg mass generated the greatest number of polymorphic markers (20) and was observed with the highest gene diversity value (14.3%). All populations, except WTX-2 generated population-specific markers. Comparison of genetic differentiation estimates, which evaluate the degree of genetic subdivision, demonstrated good agreement between  $G_{ST}$  and  $\theta$  values, 0.38 and 0.50, respectively for field populations, and 0.44 and 0.50, respectively for all populations. Average genetic divergence ( $D$ ) indicated that the WTX-1 population was the most differentiated. Average  $D$  results from the Argentina population support the taxonomic data that it is a different species. The present results estimate the population genetic structure of *G. ashmeadi*, demonstrating extensive genetic divergence and restricted gene flow ( $N_m = 0.83$ ) among populations. These results are of interest to the Pierce's Disease/Glassy-winged Sharpshooter biological control program because the key to successful biological control may not be in another species, but instead in different geographic races or biotypes.

## INTRODUCTION

*Gonatocerus ashmeadi* (Girault) (Hymenoptera: Mymaridae) is a primary egg parasitoid of *Homalodisca coagulata* (Say) (Homoptera: Cicadellidae), the glassy-winged sharpshooter (Huber 1998). A biological control program is currently in progress in California against *H. coagulata* because this xylem feeding sharpshooter is a serious economic pest that vectors a strain of *Xylella fastidiosa* (Wells), a bacterium that causes Pierce's Disease in grapevines. Studies of allele or marker frequencies in naturally occurring parasitoid populations are important, not only for identifying genetic variation of potential benefit in the selection and screening of biological control organisms, but also for the detection of genetic markers indicative of specific biological traits or geographic origins. In addition, the recognition of intraspecific variation can be as crucial for the success of biological control programs as is sound species determination. Populations of parasitoids from distinct geographical regions may differ in relevant biological characteristics of importance to biological control (Powell and Walton 1989; Narang *et al.* 1993; Unruh and Woolley 1999). An aim of genetically comparing different populations of the same species of natural enemies is to identify the strain that is most adapted to the environment where it will be released (Messenger and van den Bosch 1971); in other words, the key to successful biological control may not be in another species, but instead in different geographic races or biotypes (Diehl and Bush 1984). Reliable methods are needed for distinguishing various exotic strains of these biological control agents from those indigenous to the U. S., including parasitoids from different states within the U. S. Release of unidentified and uncharacterized strains can make it difficult to document their establishment and dispersal. Therefore, genetic typing of strains prior to their release in the field is highly desirable (Narang *et al.* 1993).

## OBJECTIVES

1. Estimate genetic variation or gene diversity within and among populations.
2. Estimate the population genetic structure.
3. Determine whether ISSR-PCR was sensitive enough to identify diagnostic markers in geographic populations.
4. Confirm the species identification of a population of egg parasitoids from Argentina identified as near *G. ashmeadi*.

## RESULTS AND CONCLUSIONS

### *ISSR-PCR Marker Heterozygosity and Genetic Diversity*

A total of 41 polymorphic markers were generated in the six populations of *G. ashmeadi* (163 individuals) from the U. S. with three pooled ISSR-PCR reactions.  $G^2$ -contingency tests indicated significant heterogeneity of marker frequency across all U. S. populations for 31 of 41 markers and for 25 of 34 markers for the field populations (not shown). All populations,

except the WTX-2, were associated with population-specific markers (data not shown). Within populations, gene diversity values ( $h$ ) were observed ranging from 2.9 to 14.3% with WTX-1 having the lowest and QFL having the highest value (Table 1). In general, the two Weslaco populations (WTX-1 and -2) were found to have the lowest  $h$  values. No significant differences in  $h$  were seen between the two Weslaco populations ( $t = 1.49$ ,  $df = 58$ ,  $P > 0.05$ ), but significant differences ( $P < 0.05$ ) were observed between WTX-1 and the rest of the U. S. populations. Interestingly, no significant differences in  $h$  were observed between the reared LA and the rest of the field populations. The fact that QFL was associated with an  $h$  value of 14.3% was surprising since this population was from a single egg mass. Overall, the field populations and all the U. S. *G. ashmeadi* populations together had an  $h$  value of 23.0 and 20.8%, respectively. The number of polymorphic markers ranged from 12 to 20 with WTX-1 and -2 having the lowest and QFL the highest. Percentage of polymorphic markers (%P) ranged from 29.3 to 58.8%, but overall, 100% of the ISSR-PCR markers were polymorphic, including the field populations analyzed separately. The two Weslaco populations were associated with the lowest %P and QFL with the highest. It is interesting to note that even though both LA and WTX-1 were reared, WTX-1 is presented with a significantly ( $P < 0.05$ ) lower  $h$  value. These results may indicate a real genetic difference between the two Weslaco populations, including the possibility of sympatric strains.

### ***ISSR-PCR Differentiation Among US G. ashmeadi Populations***

Table 2 presents the results from the different approaches used to apportion variation into within- and among-populations levels. Simultaneous exact tests for population differentiation indicated that highly significant differences in marker frequencies exist among the six U.S. populations (All:  $\chi^2 = 676.2$ ;  $df = 82$ ;  $P = 0.0000$ , and  $fc: \chi^2 = 485.2$ ;  $df = 68$ ;  $P = 0.0000$ ). These statistically significant tests suggest that discrete subpopulations exist. The average genetic diversity within populations ( $H_s$ ) value for the field populations is 14.4%. Table 2 also shows a comparison of other genetic differentiation estimates,  $G_{ST}$  and  $\theta$ . Good agreement was seen between  $G_{ST}$  and  $\theta$  values, respectively for field and for all populations. The  $G_{ST}$  values for field and all populations indicate that about 38 and 44% of the variance is distributed among populations, and 62 and 56% is distributed within populations, respectively. The  $\theta$  values show that about 50% of the variance is seen among populations in both field and all populations. The indirect estimate of gene flow,  $N_m$  based on  $G_{ST}$ , demonstrated low values for both field and all U. S. populations. These values indicate restricted gene flow among the populations.

### ***Genetic Relatedness among G. ashmeadi Populations from the US***

Average genetic divergence ( $D$ ) among both field [Nei = 0.1702 (0.1021-0.2230); Reynolds = 0.6208 (0.4069-0.8138)] and all populations [Nei = 0.1304 (0.0715-0.2024); Reynolds = 0.6512 (0.3705-0.8890)] was high (Table 3). We compared the level of genetic divergence between the field populations and the WTX-1 and LA reared populations and found mean  $D$  values of 0.1806 (Nei) and 0.8589 (Reynolds) and 0.1065 (Nei) and 0.5371 (Reynolds), respectively. These results indicate that WTX-1 is more diverged than LA. A comparison of Nei's genetic distance within the Texas populations, WTX-2 vs WTX-1 (0.1391) and WTX-2 vs SATX (0.1286), showed that divergence is slightly higher between the Weslaco populations. Sympatric species tend to have higher levels of genetic differentiation; more work is needed to confirm this possibility. The divergence between ARG and all U. S. *G. ashmeadi* populations was very high, 0.3633 (Nei) and 1.6093 (Reynolds), respectively. These results support the taxonomic data that ARG is another species. Dendrograms based on Nei's genetic distance are shown on Fig. 1 with all populations including ARG (Fig. 1A) and the field populations analyzed separately (Fig. 1B). At least two main clusters are identified on the dendrogram with ARG clustered as an outlier (Fig. 1A). Within a second cluster or all *G. ashmeadi* from the U. S., WTX-1 appears to be the most differentiated (Fig. 1A). The CA population appears to form a second subcluster and the two southeastern populations, LA and QFL form a single cluster. The WTX-1 and -2 populations are distributed in different clusters. Also within Texas (Fig. 1B), WTX-2 and SATX show divergence as they appear on a separate cluster. It is interesting to note that this same pattern of differentiation is seen with *H. coagulata* within Texas (de León *et al.* 2004).

In summary, the major observations of this study were that 1) among *G. ashmeadi* populations, based on genetic differentiation measurements (exact test,  $G_{ST}$ ,  $\theta$ ), extensive genetic structure was identified; 2) the mean expected gene diversity value for LA did not differ from field populations, whereas WTX-1 was observed with a significantly lower mean expected gene diversity value as compared to field populations (except WTX-2); 3) QFL generated the most polymorphic markers (20) with only 13 individuals, even though they were all siblings or from one egg mass. This is an interesting result since it may be assumed that siblings are not associated with high variability or have isofemale line characteristics. These results indicate that *G. ashmeadi* parasitoid siblings somehow manage to maintain their genetic diversity. Further studies are required to confirm this observation in this species and other *Gonatocerus* species. Variation within 10 male individuals (*Anaphes* sp.nov.) was demonstrated with RAPD markers by Landry *et al.* (1993), but they were not from the same egg mass; 4) based on genetic distance or average divergence, WTX-1 appeared to be the most differentiated population. Within Texas, field populations WTX-2 and SATX appeared on separate clusters, indicating that these populations are differentiated even though they are within the same state; and 5) The ARG population is confirmed to be a different species. More research is required to confirm these results, sequencing of standard genes [e. g., mitochondria cytochrome oxidase (COI)] and ITS-2 fragments are in progress.

**Table 1. Single-populations descriptive statistics for *G. ashmeadi* from the U. S. and genetic variation statistics for all loci.** Genetic variation was analyzed using the POPGENE 3.2 genetic software program and the program Tools for Population Genetic Analyses (TPPGA). No. M, number of monomorphic markers; No. P., number of polymorphic markers; %P, percentage of polymorphic loci; Poym. ratio, number of polymorphic markers per number of insects; *h*, gene diversity (SD). One-tailed unpaired *t* test performed for *h* values.

Pop.	No. Insects	No. M	No. P	Total# markers	%P	Polym. ratio	<i>h</i> (SD)
CA	30	5	16	21	39.2	0.53	0.1329 (0.182) <sup>a</sup>
WTX-1	30	7	12	19	29.3	0.40	0.0290 (0.158)
WTX-2	30	6	13	19	31.7	0.43	0.0901 (0.160)
SATX	30	5	16	21	39.0	0.53	0.1123 (0.170) <sup>a</sup>
LA	30	5	17	22	41.5	0.57	0.1252 (0.182) <sup>a</sup>
QFL	13	1	20	21	58.8	1.54	0.1431 (0.199) <sup>a</sup>
Fc	103	0	34	34	100.0	0.33	0.2300 (0.184)
All	163	0	41	41	100.0	0.25	0.2082 (0.187)
ARG	30	11	8	19	16.7	0.27	0.0434 (0.127)

<sup>a</sup>Significantly different from WTX-1,  $P < 0.05$ ;  $df = 58$

**Table 2.** Nei's analysis of gene diversity in populations of *G. ashmeadi* from the US (fc, field collected; Ht, total genetic diversity (SD); Hs, average genetic diversity within populations (SD);  $G_{ST}$  (mean), coefficient of gene differentiation;  $\theta$  (mean), theta (SD) is analogous to  $F_{ST}$ ; and Nm, gene flow).

Ht	Hs	$G_{ST}$	$\theta$	Nm
fc 0.2312 (0.032)	0.1442 (0.016)	0.3761	0.4957 (0.077)	0.8295
All 0.2087 (0.034)	0.1161 (0.013)	0.4438	0.4927 (0.057)	0.6267

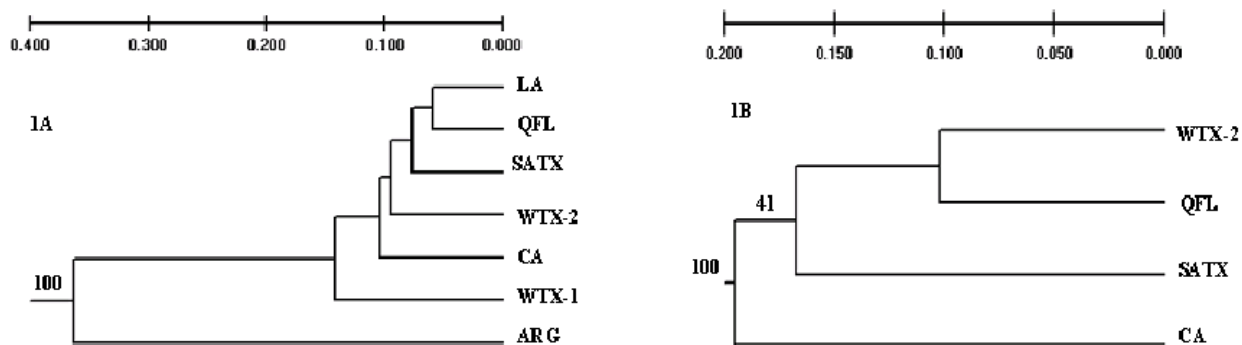
**Table 3.** Nei's unbiased (1987) genetic distance (below diagonal) and Reynolds et al. (1983) genetic distance (above diagonal). Six populations of *G. ashmeadi* from the US field populations were also analyzed separately (bottom portion of table).

Pop.	CA	WTX-1	WTX-2	SATX	LA	QFL
CA	*****	0.8682	0.6818	0.6441	0.6275	0.4227
WTX-1	0.2024	*****	0.8080	0.8703	0.6871	0.8890
WTX-2	0.1341	0.1391	*****	0.7213	0.6663	0.5322
SATX	0.1384	0.1789	0.1286	*****	0.4842	0.4956
LA	0.1422	0.1335	0.1233	0.0890	*****	0.3705
QFL	0.0896	0.2020	0.0890	0.0951	0.0715	*****

Pop.	CA	WTX-2	SATX	QFL
CA	*****	0.8138	0.8075	0.4559
WTX-2	0.2215	*****	0.7741	0.4069
SATX	0.2230	0.2015	*****	0.4666
QFL	0.1308	0.1021	0.1328	*****





**Figure 1:** Dendrograms based on Nei's genetic distance by the method of UPGMA. Relationships (A) showing the six US geographic populations of *G. ashmeadi* and a population classified as near *G. ashmeadi* (M2012) from Argentina performed by ISSR-PCR DNA fingerprinting. Field collected populations were also analyzed separately (B). Genetic distances are indicated above the dendrograms and bootstrap support values are indicated at the nodes.

## REFERENCES

- Collins FH, Paskewitz SM. 1996. A review of the use of ribosomal DNA (rDNA) to differentiate among cryptic *Anopheles* species. *Insect Molecular Biology* 5: 1-9.
- de León JH, Jones WA, Morgan DJW. 2004. Population genetic structure of *Homalodisca coagulata* (Homoptera: Cicadellidae), the vector of the bacterium *Xylella fastidiosa* causing Pierce's disease in grapevines. *Annals of the Entomological Society of America* 97: 809-818.
- Diehl, SR, Bush GL. 1984. An evolutionary and applied perspective of insect biotypes. *Annual Review of Entomology* 29: 471-504.
- Huber JT. 1998. The species groups of *Gonatocerus* Nees in North America with a revision of the *sulphuripes* and *ater* groups (Hymenoptera: Mymaridae). *Memoirs of the Entomological Society of Canada* 141: 1-109.
- Landry BS, Dextraze L, Biovin G. 1993. Random amplified polymorphic DNA markers for DNA fingerprinting and genetic variability assessment of minute parasitic wasp species (Hymenoptera: Mymaridae and Trichogrammatidae) used in biological control programs of phytophagous insects. *Genome* 36: 580-587.
- Messenger PS, van den Bosch R. 1971. The adaptability of introduced biological control agents. In: Huffaker, CB editor. *Biological Control*, 68-92. Plenum Press, New York.
- Narang SK, Leopold RA, Krueger CM, DeVault JD. 1993. Dichotomous RAPD-PCR key for identification of four species of parasitic hymenoptera. In: Narang SK, Barlett AC, Faust RM, editors. *Applications of Genetics to Arthropods of Biological Control Significance*, 53-67. CRC Press Inc, Boca Raton, Florida.
- Powell W, Walton MP. 1989. The use of electrophoresis in the study of hymenopteran parasitoids of agricultural pest. In: Loxdale HD, den Hollander J, editors. *Electrophoretic Studies on Agricultural Pests*, 443-65. Oxford, Clarendon.
- Unruh TR, Woolley JB. 1999. Molecular Methods in Classical Biological Control. In: Van Driesche RG, Bellows TS, Jr., editors. *Biological Control*, 57-85. Chapman and Hall, NY.

## FUNDING AGENCIES

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# MOLECULAR DISTINCTION BETWEEN POPULATIONS OF *GONATOCERUS MORRILLI*, EGG PARASITOID OF THE GLASSY-WINGED SHARPSHOOTER, FROM TEXAS AND CALIFORNIA

## Project Leader:

Jesse H. de León  
USDA, ARS  
Beneficial Insects Research Unit  
Weslaco, Texas 78596

## Cooperators:

Walker A. Jones  
USDA, ARS  
Beneficial Insects Research Unit  
Weslaco, Texas 78596

David J. W. Morgan  
CDFA  
Mount Rubidoux Field Station  
Riverside, California 92501

**Reporting period:** The results reported here are from work conducted from fiscal year 2003 to fiscal year 2004.

## ABSTRACT

Two molecular methods were utilized to distinguish geographic populations of *Gonatocerus morrilli* (Howard) from Texas and California and to test the possibility that this species could exist as a species-complex. Inter-Simple Sequence Repeat-Polymerase Chain Reaction (ISSR-PCR) was performed with a 5'-anchored ISSR primer. Twenty-five markers were generated with four populations (40 individuals) of *G. morrilli*, 23 were polymorphic and percentage of polymorphic loci was 92%. Most markers could be considered diagnostic since there was no band sharing between the Texas and California populations. Such differences typically are not found unless the populations are reproductively isolated. Exact tests for population differentiation indicated significant differences in markers frequencies among the populations. Comparison of other genetic differentiation estimates, which evaluate the degree of genetic subdivision, demonstrated excellent agreement between  $G_{ST}$  and  $\theta$  values, 0.92 and 0.94, respectively; indicating that about 92 to 94% of the variance was distributed among populations. Average genetic divergence ( $D$ ), as measured by genetic distance, was extremely high ( $Nei = 0.82$  and  $Reynolds = 2.79$ ). A dendrogram based on  $Nei$ 's genetic distance, separated the Texas and California populations into two clusters, respectively. Amplification of the Internal Transcribed Spacer-1 (ITS-1) region showed no size differences, whereas the ITS-2 DNA fragments varied in size between the two geographic populations. The ITS-2 fragment sizes were about 865 and 1099 base pairs for the California and Texas populations, respectively. The present study using the two molecular methods provides novel data critical to the glassy-winged sharpshooter/Pierce's disease biological control program in California.

## INTRODUCTION

*Gonatocerus morrilli* (Howard) (Hymenoptera: Mymaridae) is an egg parasitoid of *Homalodisca coagulata* (Say) (Homoptera: Cicadellidae), the Glassy-winged Sharpshooter (Turner and Pollard 1959; Triapitsyn *et al.* 1998). This primary egg parasitoid species is common in the southern United States and Mexico (Huber 1988). A biological control program is currently in progress in California against *H. coagulata*, a xylem feeding leafhopper, which is a serious economic pest that transmits a strain of *Xylella fastidiosa* (Wells), a bacterium that causes Pierce's disease in grapevines (*Vitis vinifera* L. and *V. labrusca* L.) (Hopkins and Mollenhauer 1973). Accurate identification of natural enemies is critical to the success of classical biological control programs. Lack of proper identification procedures has affected the early stages of several projects (Messing and Aliniaze 1988; Löhr *et al.* 1990). There is a need for molecular markers for natural enemies to provide new characters for studies of phylogenetic relatedness, for identification of cryptic species and biotypes, and for the assessment of heritable variation for population genetics and ecological investigations (Unruh and Woolley 1999). Studies of allele or marker frequencies in naturally occurring parasitoid populations are important, not only for identifying genetic variation of potential benefit, but also for the detection of genetic markers indicative of specific biological traits or geographic origins. Furthermore, the recognition of intraspecific variation can be as crucial for the success of biological control programs as is sound species determination (Powell and Walton 1989; Narang *et al.* 1993; Unruh and Woolley 1999).

## OBJECTIVES

1. Survey molecular methods useful in egg parasitoid identification and discrimination
2. Investigate the possibility that *G. morrilli* could exist as a species-complex in nature

## RESULTS AND CONCLUSIONS

### ISSR-PCR DNA Fingerprinting.

Figure 1 shows an example of ISSR-PCR DNA fingerprinting demonstrating the banding pattern differences between the geographic populations of *G. morrilli* from California (OrCo) and Texas (Wes-2) performed with a 5'-anchored ISSR primer. Markers ranged in size from about 200 to 900 base pairs. Overall, a total of 25 markers were generated among all four populations with a total of 40 individuals. Twenty-three were polymorphic and percentage of polymorphic loci was 92%. Within individual populations, no diversity was seen within the California populations and only slight diversity was observed in the Texas populations. For the Texas populations, Wes-2 and Wes-3, 5 polymorphic markers each were generated and 20% of the markers were polymorphic. Most markers are geographic-specific and can therefore be considered diagnostic since there is no band sharing between the Texas and California populations.

### **ISSR-PCR Differentiation Among Four *G. morrilli* Populations.**

Exact tests (simultaneous analysis) for population differentiation indicated that highly significant differences in marker frequencies existed among the *G. morrilli* populations (Table 1). Total genetic diversity ( $H_t$ ) was high (35%), whereas the average genetic diversity within populations was low (3%). Table 1 also shows a comparison of other genetic differentiation estimates,  $G_{ST}$  and  $\theta$ , which evaluate the degree of genetic subdivision among populations. Excellent agreement was seen between  $G_{ST}$  and  $\theta$  values, 0.92 and 0.94, respectively. These values indicate that about 92 to 94% of the variance is distributed among populations. The indirect estimate of gene flow,  $N_m$  based on  $G_{ST}$ , demonstrated a low value (0.04) among the geographic populations; this value indicates highly restrictive gene flow. Overall, genetic differentiation measurements (exact tests,  $G_{ST}$ ,  $\theta$ , and  $N_m$ ) indicate profound genetic divergence/structuring between *G. morrilli* populations from Texas and California.

### **Genetic Relatedness Among *G. morrilli* Populations.**

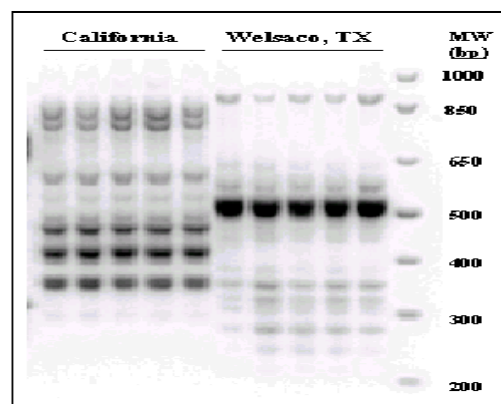
Levels of genetic divergence among populations were also determined by calculating pairwise estimates for genetic distance by the procedures of Nei (1978) and Reynolds *et al.* (1983) (Table 2). Average genetic divergence ( $D$ ) among populations was extremely high [ $Nei = 0.82$  (0.89-1.07) and  $Reynolds = 2.79$  (1.4-3.4)]. A dendrogram based on Nei's genetic distance is shown on Fig. 2 with all *G. morrilli* geographic populations. Two clades are identified on the dendrogram with the California and Texas populations appearing on separate clusters. These two clusters are supported by strong bootstrap support values, 68 and 64%, respectively for the California and Texas populations.

### **Amplification of the ITS-1 and -2 regions in *G. morrilli* Geographic Populations.**

Monomorphic patterns were demonstrated with amplification of the ITS-1 region in all of the populations from California and Texas (~850 bp) (Fig. 3); whereas, polymorphic or different DNA fragment sizes were detected within the ITS-2 region. The California populations were observed with an ITS-2 fragment size of about 865 base pairs and the Texas populations with a size of about 1099 base pairs.

Good agreement is seen between the two molecular methods and they both suggest that cryptic species may exist. The results with ISSR-PCR demonstrating distinct banding patterns (no band sharing) between geographic populations typically is not found unless the populations are reproductively isolated. Similar results were obtained by Hoy *et al.* (2000) with two populations of *Ageniaspis citrocola* performed by RAPD-PCR. The following genetic differentiation parameters, exact test,  $G_{ST}$ ,  $\theta$ , genetic distances, and gene flow ( $N_m$ ) lend support to this observation. The extremely low value for gene flow between the populations from California and Texas lend support that these populations are isolated reproductively. Restricted gene flow usually leads to increased differentiation among populations as seen from the  $G_{ST}$  and  $\theta$  values (92 to 94% of the variance is seen among populations). In addition, the divergence ( $D$ ) between these populations is also high.

Methods incorporating SSR appear to be sensitive at detecting DNA polymorphisms in natural populations. Previously, we utilized ISSR-PCR to distinguish three species of *Homalodisca* sharpshooters (*H. coagulata*, *H. liturata*, and *H. insolita*) (de León and Jones 1994). Even though this method is sensitive, there are not many reports in the literature utilizing ISSR-PCR to study insect population genetics and phylogenetics. We have also had success determining the population genetic structure of *H. coagulata* representing 19 populations from through the U. S. (de León *et al.* 2004). The Internal Transcribed Spacer regions (ITS-1 and -2) have been used extensively to examine the taxonomic status of species and for diagnostic purposes, and success with this approach has been reviewed by Collins and Paskewitz (1996). Stouthamer *et al.* (1999) used ITS-2 DNA fragment sizes as taxonomic characters to develop a precise identification key for sibling species of the genus *Trichogramma*. In cases where species were observed with similar sized ITS fragments these authors suggested amplification, sequencing, and restriction digestion.



**Figure 1.** Representative example of ISSR-PCR DNA fingerprinting of *G. morrilli* populations from California and Texas. Reactions were performed with genomic DNA from separate individuals and the 5'-anchored ISSR primer HVH(TG)<sub>7</sub>T (Zietkiewicz *et al.* 1994) as describe in the Materials and Methods. M: 1.0 Kb Plus DNA Ladder.

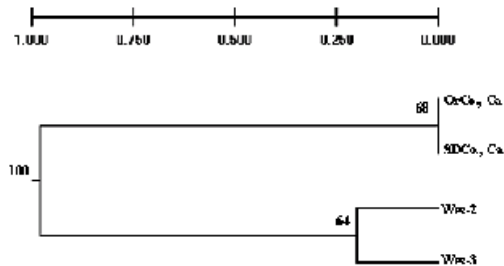
These novel observations strongly suggest that *G. morrilli* may exist in nature as a species-complex. Results from our recent study with *H. coagulata* suggest that a subset of these insects have their origin in Texas (de León *et al.* 2004). Those results together with our present results with *G. morrilli* may suggest that this egg parasitoid from Texas may be a good candidate for the biological control efforts in California against *H. coagulata*, the causative agent of Pierce’s disease.

**Table 1. Nei’s analysis of gene diversity in populations of *G. morrilli* from Texas and California.** Ten individuals per population (40 total) were subjected to ISSR-PCR DNA fingerprinting. Genetic variation was analyzed using the POPGENE 3.2 genetic software program and the program Tools for Population Genetic Analyses (TPFGA).  $\chi^2$ , exact tests (simultaneous analysis) for population differentiation, df = degrees of freedom; Ht, total genetic diversity (SD), Hs, average genetic diversity within populations (SD);  $G_{ST}$  (mean), coefficient of gene differentiation;  $\theta$  theta (analogous to  $F_{ST}$ ), and Nm, gene flow. \*\*\* $P = 0000$ .

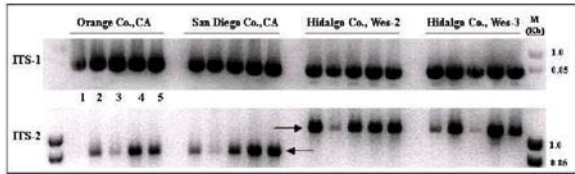
$\chi^2$ (df)	Ht	Hs	$G_{ST}$	$\theta$	Nm
400.8 (50)***	0.35	0.03 (0.04)	0.92 (0.00)	0.94	0.04 (0.02)

**Table 2. Nei’s unbiased (1978) genetic distance (below diagonal) and Reynolds et al. (1983) genetic distance (above diagonal).** Four geographic populations of *G. morrilli*, two from Texas (Hidalgo Co, Wes-2 and Wes-3) and two from California (OrCo, Orange county and SDCo, San Diego county).

Pop	OrCo	SDCo	Wes-2	Wes-3
OrCo	***	undef	3.40	2.88
SDCo	0.00	***	3.40	2.88
Wes-2	1.07	1.07	***	1.40
Wes-3	0.89	0.89	0.20	***



**Figure 2.** Dendrogram based on Nei’s genetic distance (1978) by the method of UPGMA. Relationships among the four geographic populations of *G. morrilli* performed by ISSR-PCR DNA fingerprinting. Genetic distances are indicated above the dendrograms and bootstrap support values are indicated at the nodes.



**Figure 3.** Amplification of the Internal Transcribed Spacer regions (ITS). The ITS-1 and –2 regions were amplified with standard ITS-specific primers with genomic DNA from five separate individuals from each geographic population. Arrows indicate different ITS fragment sizes. M: 1.0 Kb Plus DNA Ladder.

## REFERENCES

- Collins FH, Paskewitz SM. 1996. A review of the use of ribosomal DNA (rDNA) to differentiate among cryptic *Anopheles* species. *Insect Molecular Biology* 5: 1-9.
- de León JH, Jones WA. 2004. Detection of DNA polymorphisms in *Homalodisca coagulata* (Homoptera: Cicadellidae) by polymerase chain reaction-based DNA fingerprinting methods. *Annals of the Entomological Society of America* 97: 574-585.
- de León JH, Jones WA, Morgan DJW. 2004. Population genetic structure of *Homalodisca coagulata* (Homoptera: Cicadellidae), the vector of the bacterium *Xylella fastidiosa* causing Pierce's disease in grapevines. *Annals of the Entomological Society of America* 97: 809-818.
- Hopkins DL, Mollenhauer HH. 1973. Rickettsia-like bacterium associated with Pierce's disease of grapes. *Science* 179: 298-300.
- Hoy MA, Ayyamperumal J, Morakete R, Lo MKC, Nguyen R. 2000. Genomic analyses of two populations of *Ageniaspis citricola* (Hymenoptera: Encyrtidae) suggest that a cryptic species may exist. *Biological Control* 17: 1-10.
- Huber JT. 1998. The species groups of *Gonatocerus* Nees in North America with a revision of the *sulphuripes* and *ater* groups (Hymenoptera: Mymaridae). *Memoirs of the Entomological Society of Canada* 141: 1-109.
- Löhr BA, Varela M, Santos B. 1990. Exploration for natural enemies of the cassava mealybug, *Phenococcus manihoti* (Homoptera: Pseudococcidae), in South America for the biological control of this introduced pest in Africa. *Bulletin of Entomological Research* 80: 417-425.
- Messing RH, Aliniaze MT. 1988. Hybridization and host suitability of two biotypes of *Trioxys pallidus* (Hymenoptera: Aphididae). *Annals of the Entomological Society of America* 81: 6-9.
- Narang SK, Leopold RA, Krueger CM, DeVault JD. 1993. Dichotomus RAPD-PCR key for identification of four species of parasitic hymenoptera. In: Narang SK, Barlett AC, Faust RM, editors. *Applications of Genetics to Arthropods of Biological Control Significance*, 53-67. CRC Press Inc, Boca Raton, Florida.
- Nei M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* 89: 583-590.
- Powell W, Walton MP. 1989. The use of electrophoresis in the study of hymenopteran parasitoids of agricultural pest. In: Loxdale HD, den Hollander J, editors. *Electrophoretic Studies on Agricultural Pests*, 443-65. Oxford, Clarendon.
- Reynolds J, Weir BS, Cockerham CC. 1983. Estimation of the coancestry coefficient: basis for a short-term genetic distance. *Genetics* 105: 767-779.
- Stouthamer R, Hu J, Van Kan FJPM, Platner GR, Pinto JD. 1999. The utility in internally transcribed spacer 2 DNA sequences of the nuclear ribosomal gene for distinguishing sibling species of *Trichogramma*. *BioControl* 43: 421-440.
- Triapitsyn SV, Mizell RF III, Bossart JL, Carlton CE. 1998. Egg parasitoids of *Homalodisca coagulata* (Homoptera: Cicadellidae). *Florida Entomologist* 81: 241-243.
- Turner WF, Pollard HN. 1959. Life histories and behavior of five insect vectors of phony peach disease. *USDA Technical Bulletin* 1188: 28 pp.
- Unruh TR, Woolley JB. 1999. Molecular Methods in Classical Biological Control. In: Van Driesche RG, Bellows TS, Jr., editors. *Biological Control*, 57-85. Chapman and Hall, NY.
- Zietkiewicz E, Rafalski A, Labuda D. 1994. Genomic fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* 20:176-183.

## FUNDING AGENCIES

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# SEQUENCE DIVERGENCE IN TWO MITOCHONDRIAL GENES (COI AND COII) AND IN THE ITS2 RDNA FRAGMENT IN GEOGRAPHIC POPULATIONS OF *GONATOCERUS MORRILLI*, A PRIMARY EGG PARASITOID OF THE GLASSY-WINGED SHARPSHOOTER

## Project Leader:

Jesse H. de León  
USDA, ARS  
BIRU  
Weslaco, TX 78596

## Cooperators:

Walker A. Jones  
USDA, ARS  
BIRU  
Weslaco, TX 78596

David J. W. Morgan  
CDFA  
Mount Rubidoux Field Station  
Riverside, CA 92501

Russell F. Mizell, III  
University of Florida  
Quincy, FL 32351

**Reporting period:** The results reported here are from work conducted from fiscal year 2003 to fiscal year 2004.

## ABSTRACT

The aim of the present study was to resolve the genetic relationships of geographic populations of *Gonatocerus morrilli*, a primary egg parasitoid of the Glassy-winged Sharpshooter. A phylogenetic approach was implemented by sequencing two mitochondrial genes (COI and COII) and the Internal Transcribed Spacer-2 (ITS2) region of several individuals per population. Two populations from Weslaco, TX (WTX) (collected at different times), one from Quincy, FL (QFL), two from California (CA) (Orange and San Diego counties), and an outgroup (*G. ashmeadi*) were analyzed. For all three sequence fragments, percentage sequence divergence (%D) (as measured by genetic distance), the results demonstrated that both the WTX and QFL populations were closely related; in contrast, the %D between WTX and CA fell within the range of the outgroup, *G. ashmeadi*. For all three sequence fragments, Neighbor-Joining distance trees separated the CA and WTX and QFL populations into two distinctive clades (A and B). The topology of the clades in each case was supported by very strong bootstrap values, 100% in the three sequence fragments (COI, COII, and ITS2). The present molecular phylogenetics results provide strong evidence that *G. morrilli* from California may be a different species. The findings of the present study are important to the Glassy-winged Sharpshooter/Pierce's disease biological control program in California.

## INTRODUCTION

*Gonatocerus morrilli* (Howard) (Hymenoptera: Mymaridae) is an egg parasitoid of *Homalodisca coagulata* (Say) (Homoptera: Cicadellidae), the Glassy-winged Sharpshooter (Turner and Pollard 1959; Triapitsyn *et al.* 1998). This primary egg parasitoid species is common in the southern United States and Mexico (Huber 1988). A biological control program is currently in progress in California against *H. coagulata*, a xylem feeding leafhopper, which is a serious economic pest that transmits a strain of *Xylella fastidiosa* (Wells), a bacterium that causes Pierce's disease in grapevines (*Vitis vinifera* L. and *V. labrusca* L.) (Hopkins and Mollenhauer 1973). Accurate identification of natural enemies is critical to the success of classical biological control programs. Lack of proper identification procedures has affected the early stages of several projects (Messing and Aliniaze 1988; Löhr *et al.* 1990).

## OBJECTIVES

Determine the phylogenetic relationships of geographic populations of *G. morrilli* by sequencing two mitochondrial genes (COI and COII) and one rDNA spacer region (ITS2).

## RESULTS AND CONCLUSIONS

Sequence divergence in the mitochondrial COI gene in *G. morrilli* geographic populations. Levels of genetic divergence in the mtCOI gene among populations were determined by calculating the pairwise estimates for genetic distance. Recently, we determined that populations of *G. morrilli* from California and Texas shared no ISSR-PCR banding patterns, indicating that these populations were reproductively isolated. In addition, we demonstrated that the ITS2 rDNA fragments varied in size between these geographic populations (de León *et al.* 2004). The percentage sequence divergence (%D) for mtCOI is shown on Table 1. In general, the intra-population variation (0.0-0.6%) was small within each population and species, with the exception of the Quincy, FL population (QFL) (2.0-4.8%). The %D between Weslaco, TX (WTX) and QFL is 0.0-4.8%, which falls within the intra-population variation of these populations; these results indicate that these geographic populations are genetically similar. In contrast, the %D of WTX and CA is 5.4-5.6%, falling within the range (5.4-6.9%) of the outgroup (*G. ashmeadi*). The Neighbor-Joining distance tree in Fig. 1 demonstrates that the CA and WTX and QFL populations cluster into two distinctive clades (A and B). These clades are supported by very strong bootstrap values (100%).

Sequence divergence in the mitochondrial COII gene in *G. morrilli* geographic populations. The percentage sequence divergence (%D) for mtCOII is shown on Table 2. Intra-population variation is seen in both the WTX (0.0-4.5%) and QFL (0.0-3.2%) populations. The %D between WTX and QFL is 0.3-4.7%, these values fall within the intra-population variation range and therefore these populations would be considered closely related. On the other hand, the %D between WTX and CA is 7.4-11.1%, these values fall within the range (7.4-11.5%) of the outgroup (*G. ashmeadi*). The Neighbor-Joining distance tree in Fig. 2 demonstrates that the CA and WTX and QFL populations cluster into two distinctive clades (A and B). These clades are supported by very strong bootstrap values (100%).

*Sequence divergence in ITS rDNA fragment in G. morrilli geographic populations.* The percentage sequence divergence (%D) for ITS2 is shown on Table 3. The %D between WTX and QFL is 0.0-1.40%, this falls within the intra-population range of both populations and therefore shows that these populations are closely related. In contrast, the %D between WTX and CA is 6.2-10.7%, falling within the range (7.9-13.3%) of the outgroup (*G. ashmeadi*). The Nieghbor-Joining distance tree in Fig. 3 demonstrates that the CA and WTX and QFL populations cluster into two distinctive clades (A and B). These clades are supported by very strong bootstrap values (100%).

**Table 1 (COI) and Table 2 (COII).** Pairwise sequence distances (range) of mitochondrial COI and II genes from geographic populations of *G. morrilli* showing percentage divergence. The alignment program ClutstalW (Thomas *et al.* 1994) from DNASTar was utilized for these analyses. To account for intra- and inter-population variation, several individuals (3-6) were included. WTX, Weslaco, TX (two populations from Hidalgo Co; 5-6 total individuals); QFL, Quincy, FL (3 individuals); CA, California (two populations, Orange Co. and San Diego Co.; 6 total individuals); Ga, *G. ashmeadi* (outgroup) (3 individuals).

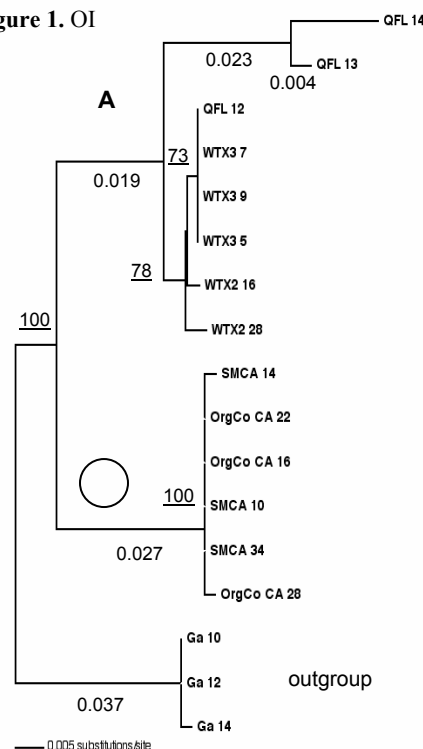
**Table 1. COI.**

Pop	WTX	QFL	CA	Ga
WTX	0.0-0.6			
QFL	0.0-4.8	2.0-4.8		
CA	5.4-5.6	5.4-8.6	0.0-0.2	
Ga	5.4-6.9	5.4-10.8	6.7-7.1	0.0-0.2

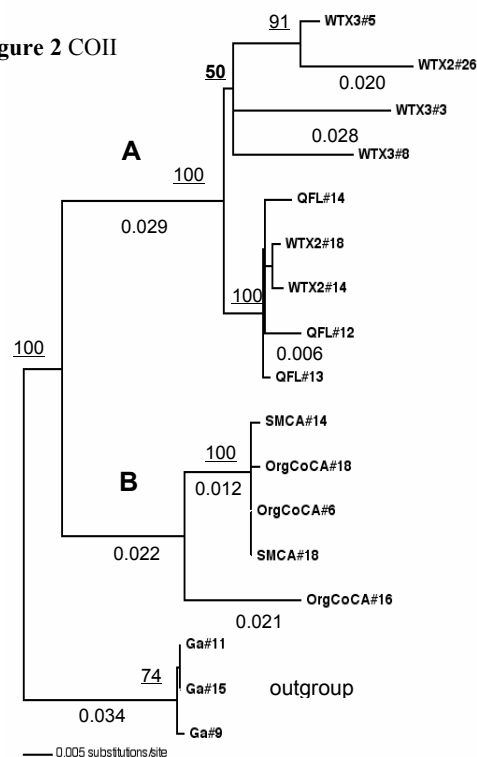
**Table 2. COII.**

Pop	WTX	QFL	CA	Ga
WTX	0.3-4.50			
QFL	0.3-4.70	0.2-0.6		
CA	7.4-11.1	7.6-8.9	0.0-3.2	
Ga	7.4-10.5	7.1-7.8	6.9-8.0	0.0-0.2

**Figure 1. OI**



**Figure 2 COII**

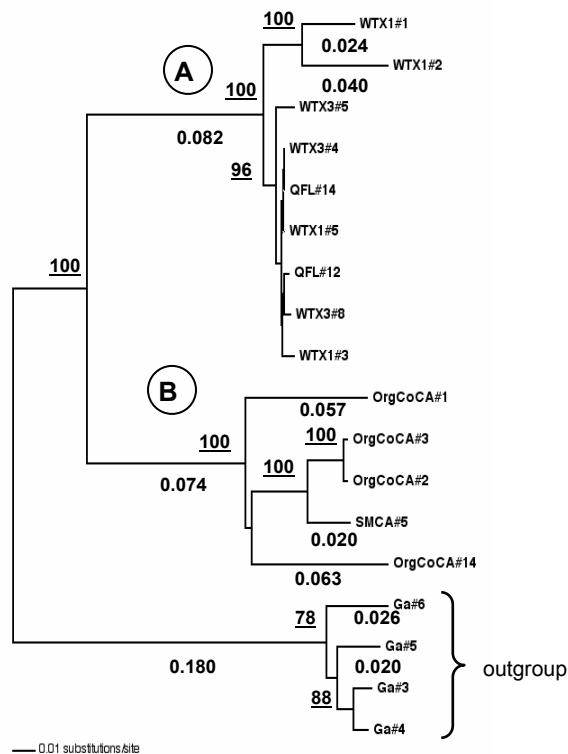


**Figure 1 (COI) and Figure 2 (COII).** Phenograms of mitochondrial COI and COII genes from geographic populations of *G. morrilli*. Analyses were performed with the alignment program ClustalX (Thompson *et al.* 1997) and the Nieghbor-Joining trees were created with the phylogenetic program PAUP 4.0 (Swofford 2002). In the genetic distance trees *G. ashmeadi* are included as an outgroup, displaying branch lengths (below branches) and bootstrap values (above branches underlined), as percentage of 1000 replications. To account for intra- and inter-population variation, several randomly chosen individuals (3-6) were included. SMCA, San Marcos, CA; OrgCo CA; Orange county, California.



**Table 3. Pairwise sequence distances (range) of ITS-2 rDNA fragments from geographic populations of *G. morrilli* showing percentage divergence.** The alignment program ClutstalW (Thomas *et al.* 1994) from DNASTar was utilized for this analysis. To account for intra- and inter-populational variation, several individuals (2-7) were included. WTX, Weslaco, TX (two populations from Hidalgo Co; 7 total individuals); QFL, Quincy, Florida (2 individuals); CA, California (two populations, Orange Co. and San Diego Co; 5 total individuals) Ga, *G. ashmeadi* (outgroup) (4 individuals).

Pop	WTX	QFL	CA	Ga
WTX	0.0-1.70			
QFL	0.0-1.40	0.0-0.30		
CA	6.2-10.7	6.3-7.70	0.0-0.60	
Ga	7.9-13.3	8.4-12.4	7.8-12.0	0.5-0.9



**Figure 3.** Phenogram of ITS2 rDNA sequence fragment from geographic populations of *G. morrilli*. Analysis was performed with the alignment program ClustalX (Thompson *et. al.* 1997) and the Nieghbor-Joining tree was created with the phylogenetic program PAUP 4.0 (Swofford 2002). In the genetic distance trees *G. ashmeadi* are included as an outgroup, displaying branch lengths (below branches) and bootstrap values (above branches underlined), as percentage of 1000 replications. To account for intra- and inter-populational variation, several randomly chosen individuals (2-7) were included. SMCA, San Marcos, CA; OrgCo CA; Orange county, California.

### REFERENCES

de León JH, Jones WA, Morgan DJW. 2004. Molecular distinction between populations of *Gonatocerus morrilli*, egg parasitoids of the glassy-winged sharpshooter *Homalodisca coagulata*, form Texas and California: Do cryptic species exist? *Journal of Insect Science* (in press).

Hopkins DL, Mollenhauer HH. 1973. Rickettsia-like bacterium associated with Pierce’s disease of grapes. *Science* 179: 298-300.

Huber JT. 1998. The species groups of *Gonatocerus* Nees in North America with a revision of the *sulphuripes* an *ater* groups (Hymenoptera: Mymaridae). *Memoirs of the Entomological Society of Canada* 141: 1-109.

Löhr BA, Varela M, Santos B. 1990. Exploration for natural enemies of the cassava mealybug, *Phenococcus manihoti* (Homoptera: Pseudococcidae), in South America for the biological control of this introduced pest in Africa. *Bulletin of Entomological Research* 80: 417-425.

Messing RH, Aliniaze MT. 1988. Hybridization and host suitability of two biotypes of *Trioxys pallidus* (Hymenoptera: Aphididae). *Annals of the Entomological Society of America* 81: 6-9.



- Thompson JD, Gibson TJ, Plewniak F, Higgins DG. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 24: 4876-4882.
- Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22:4673-4680.
- Triapitsyn SV, Mizell RF III, Bossart JL, Carlton CE. 1998. Egg parasitoids of *Homalodisca coagulata* (Homoptera: Cicadellidae). *Florida Entomologist* 81: 241-243.
- Turner WF, Pollard HN. 1959. Life histories and behavior of five insect vectors of phony peach disease. *USDA Technical Bulletin* 1188: 28 pp.
- Swofford DL. 2002. PAUP\*. Phylogenetic Analysis Using Parsimony (\*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

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# DEVELOPMENT OF MOLECULAR DIAGNOSTIC MARKERS FOR *HOMALODISCA* SHARPSHOOTERS PRESENT IN CALIFORNIA TO AID IN THE IDENTIFICATION OF KEY PREDATORS

## Project Leaders:

Jesse H. de León  
USDA, ARS  
BIRU  
Weslaco, Texas 78596

James Hagler  
USDA, ARS  
Western Cotton Res. Lab  
Phoenix, AZ 85040

Valerie Fournier & Kent Daane  
Division of Insect Biology  
University of California  
Berkeley, CA 94720

## Cooperator:

Walker A. Jones  
USDA, ARS  
BIRU  
Weslaco, TX 78596

**Reporting period:** The results reported here are from work conducted from fiscal year 2003 to fiscal year 2004.

## ABSTRACT

The aim of the present study was to develop molecular diagnostic markers to identify key predators of *Homalodisca* sharpshooter species present in California, *H. coagulata* (Glassy-winged Sharpshooter, GWSS) and *H. liturata* (Smoke-tree Sharpshooter, STSS). RAPD-PCR DNA fingerprinting of several sharpshooter species identified specific bands that were excised, sequenced, and SCAR (Sequenced Characterized Amplified Region) markers were designed. The results demonstrated that both GWSS- and *Homalodisca*-specific markers were specific toward their targets. The GWSS-specific markers amplified only GWSS and the *Homalodisca*-specific markers amplified only GWSS and STSS. The sensitivity limits for both marker sets was at 50 pg of DNA. The mitochondrial cytochrome oxidase subunit gene II (COII)-specific markers that were developed were each specific for GWSS and *Homalodisca* sharpshooters. The development of diagnostic markers designed toward *Homalodisca* sharpshooters present in California should aid in finding key predators and therefore enhance biological control efforts against these sharpshooters.

## INTRODUCTION

The Glassy-winged Sharpshooter, *Homalodisca coagulata* (Say) (Homoptera: Cicadellidae), is a large xylem feeding leafhopper that is a serious pest because it vectors a strain of *Xylella fastidiosa* (Wells), a bacterium that causes Pierce's disease in grapevines (*Vitis vinifera* and *V. labrusca*) (Hopkins and Mollenbauer 1973). A biological control program is currently in progress in California against *H. coagulata*. Effective control of GWSS will require an area-wide pest management approach. A major component of such an approach is the exploitation of the pest's natural enemies, which, when utilized to their greatest potential, can increase the effectiveness of other control tactics. Unfortunately, very little is known about GWSS natural enemies, this is especially true for their predators (Triapitsyn *et al.* 1998). Direct visual field observations of predation are difficult to obtain and historically, the study of insect predation has relied mainly on inexact and indirect techniques for measurement and analysis. Presently, Hagler and Naranjo (1997) and Hagler *et al.* (1991) have had success in developing monoclonal antibodies and detecting prey in predator gut contents by enzyme linked immunoassays (ELISA). Recently, other methods have been developed that allow for the detection of prey in predator gut contents. These molecular methods include, Sequence Characterized Amplified Region (SCAR), where RAPD-PCR species-specific bands are excised from gels, sequenced, and primers are designed toward those DNA fragments (Agusti *et al.* 1999; Agusti *et al.* 2000) and targeting genes that are present in the cell in high copy number, such as, mitochondrial genes (COI and COII) and Internal Transcribed Spacer regions (ITS1) (Agusti *et al.* 2003; Chen *et al.* 2000; Symondson 2002).

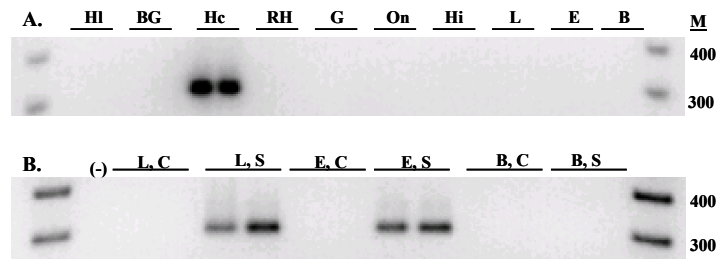
## OBJECTIVE

Develop molecular diagnostic markers for *Homalodisca* sharpshooter species (GWSS and STSS) found in California in order to identify key predators.

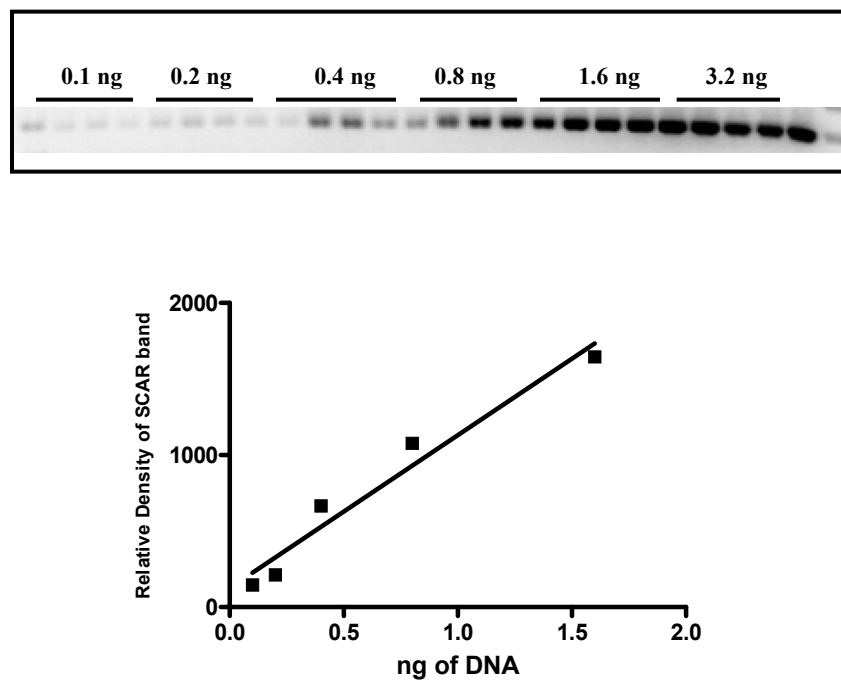
## RESULTS AND CONCLUSIONS

### *GWSS-specific SCAR (5/7) Markers*

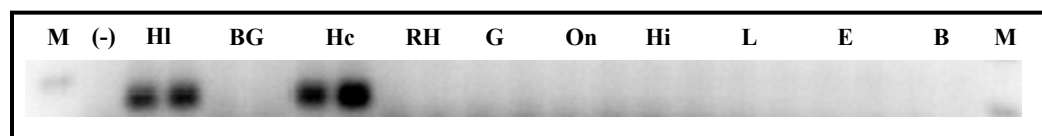
RAPD-PCR DNA fingerprinting was performed with several sharpshooter species and *Homalodisca*-specific bands were excised, sequenced, and primers designed (SCAR markers). Figure 1A demonstrates that GWSS-specific SCAR (5/7) markers were highly specific with no amplification of any other sharpshooter species or predators. The GWSS-specific markers were also able to detect GWSS eggs in predator gut contents (Figure 1B). The sensitivity of the SCAR marker set was tested by varying the amount GWSS DNA (0.1 to 3.2 ng) (Figure 2). In this experiment, the limit of sensitivity was at 100 pg, but later experiments showed the detection limit at 50 pg (not shown).



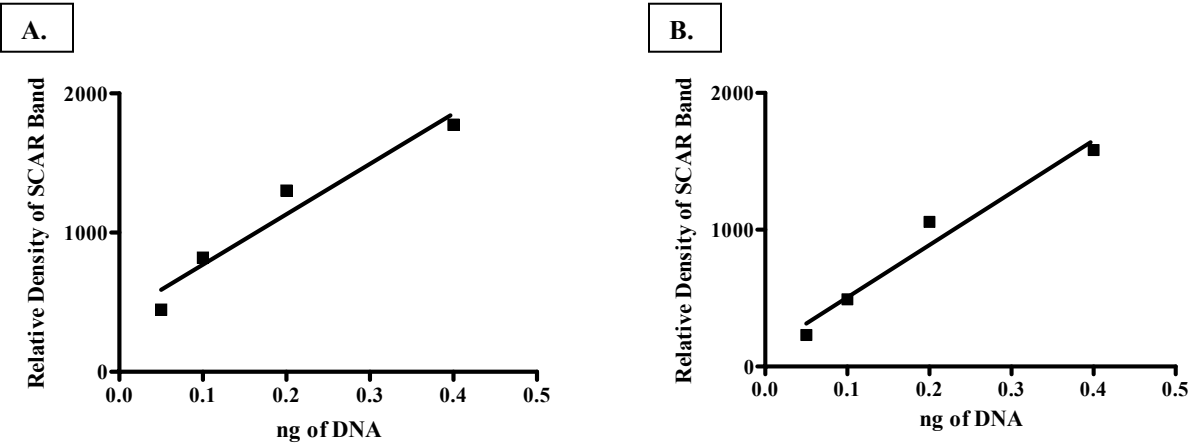
**Figure 1.** RAPD-PCR DNA fingerprinting was performed with the following sharpshooters: *Homalodisca liturata* (HI); *Graphocephala atropuncta* [blue-green (BG)]; *H. coagulata* (Hc); *Carneocephala fulgida* [red-headed (RH)]; *Draeculacephala minerva* [green (G)]; *Oncometopia nigricans* (On); and *H. insolita* (Hi). Amplification products/bands unique to GWSS were excised, sequenced, and primers (SCAR markers) were designed to amplify a 302-bp fragment. **A.** Specificity of GWSS-specific SCAR-5/7 markers. L, lacewing larvae (*Chrysoperla carnea*); E, earwig (*Forficula auricularia*); and B, ground beetle (*Calosoma sp.*). **B.** Detection of GWSS in predator gut contents by SCAR-PCR assays. (-), negative control (no template); C, control (not fed on GWSS); S, sample (fed on GWSS). Lacewing and earwig fed on GWSS eggs and ground beetle fed on a GWSS adult.



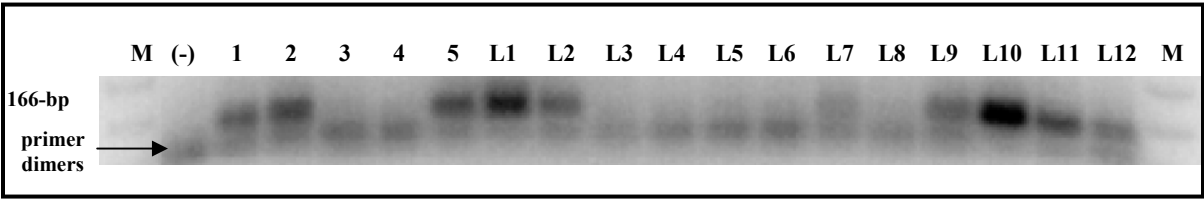
**Figure 2.** Sensitivity assay with GWSS-specific SCAR 5/7. GWSS DNA was varied from 0.1 to 3.2 ng, each point in quadruplicate (inset). The four determinations per point were averaged and plotted vs relative density of the SCAR bands. Since the highest amount of DNA (3.2 ng) did not fall within the linear portion of the curve (saturated) it was eliminated.



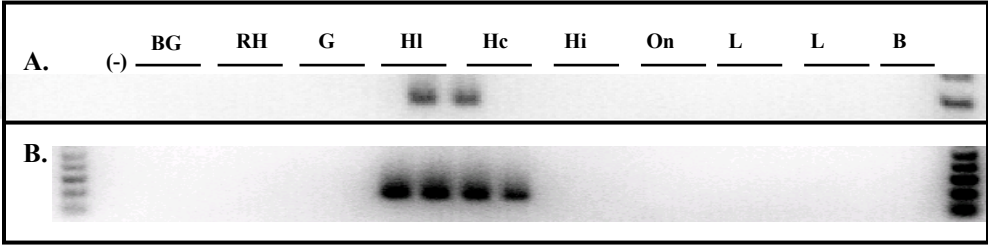
**Figure 3.** California *Homalodisca* (GWSS/STSS)-specific SCAR 6/9 specificity assay. California *Homalodisca*-specific primers were designed toward a RAPD-PCR fragment. Refer to Figure 1 for assignments.



**Figure 4.** SCAR 6/9 sensitivity assays with GWSS DNA (A) and STSS DNA (B). DNA ranged from 0.05 to 0.80 ng with each point in triplicate. The three determinations per point were averaged and plotted vs relative density of the SCAR bands. The highest amount of DNA (0.80 ng) was not in the linear portion of the curve (saturated), so it was eliminated from the analysis.



**Figure 5.** SCAR-PCR (6/9) assays with predators (Lacewing, L1-10) that fed on GWSS eggs. Lanes: 1, Qiagen prep control plus GWSS DNA; 2, crude extract control plus GWSS DNA; 3, crude extract negative control (not fed); 4, Qiagen prep negative control (not fed); 5, GWSS DNA positive control.



**Figure 6 (below).** California *Homalodisca* mitochondrial COII-specific primers. The mitochondrial COII genes of both GWSS and STSS were sequenced and both *Homalodisca*- and GWSS-specific primers were designed. Refer to fig. 1 for assignments.

***Homalodisca* (GWSS/STSS)-Specific SCAR (6/9) Markers**

Figure 3 shows the specificity of the *Homalodisca* markers, as seen only GWSS and STSS DNA is amplified with this marker set and no other sharpshooters or predators amplified. The sensitivity of this SCAR (6/9) marker set was tested with

both GWSS (Figure 4A) and STSS (Figure 4B) DNA individually. The amount of DNA was varied from 0.05 to 0.80 ng. These experiments show the sensitivity limits with both GWSS and STSS DNA to be at 50 pg. The SCAR (6/9) marker set was tested with predators (Lacewings L1-12) that fed on GWSS eggs (Figure 5). At least 7 of the 12 specimens tested positive with this marker set. The assay system was tested for competition or interference of predator DNA with both Qiagen preps and crude DNA extracts. The DNA crude extract procedure was developed as a rapid method to assay hundreds of samples more efficiently. The results show that predator DNA does not compete or interfere with the SCAR-PCR assays.

#### ***Homalodisca* and GWSS-specific Mitochondrial COII primers**

Mitochondrial DNA is present in hundreds or multiple copies within each cell (Chen *et al.* 2000; Symondson 2002). In order to increase the sensitivity of our diagnostic assays, the mtCOII genes of both GWSS and STSS were sequenced and both *Homalodisca*- and GWSS-specific primers were designed. Figure 6 demonstrates that both GWSS- (Figure 6A) and *Homalodisca*- (Figure 6B) specific primers were successful without amplifying any other sharpshooters or predators.

#### **REFERENCES**

- Agusti N, De Vicente MC, Gabarra R. 1999. Development of sequence amplified characterized region (SCAR) markers of *Helicoverpa armigera*: a new polymerase chain reaction-based technique for predator gut analysis. *Molecular Ecology* 8: 1467-1474.
- Agusti N, De Vicente MC, Gabarra R. 2000. Developing SCAR markers to study predation on *Trialeurodes vaporariorum*. *Insect Molecular Biology* 9: 263-268.
- Agusti N, Shayler SP, Harwood JD, Vaughan IP, Sunderland KD, Symondson WOC. 2003. Collembola as alternative prey sustaining spiders in arable ecosystems: prey detection withing predators using molecular markers. *Molecular Ecology* 12: 3467-3475.
- Chen Y, Giles KL, Payton ME, Greenstone MH. 2000. Identifying key cereal aphid predators by molecular gut analysis. *Molecular Ecology* 9: 1887-1898.
- de León JH, Jones WA. 2004. Detection of DNA polymorphisms in *Homalodisca coagulata* (Homoptera: Cicadellidae) by polymerase chain reaction-based DNA fingerprinting methods. *Annals of the Entomological Society of America* 97: 574-585.
- Hagler JR, Naranjo SE. 1997. Measuring the sensitivity of an indirect predator gut content ELISA: Detectability of prey remains in relation to predator species, temperature, time, and meal size. *Biological Control* 9:112-119.
- Hagler JR, Cohen AC, Enriquez FJ, Bradley-Dunlop D. 1991. An egg-specific monoclonal antibody to *Lygus hesperus*. *Biological Control* 1:75-80.
- Hopkin, D L, Mollenhauer HH. 1973. Rickettsia-like bacterium associated with Pierce's disease of grapes. *Science* 179: 298-300.
- Symondson WOC. 2002. Molecular identification of prey in predator diets. *Molecular Ecology* 11: 627-641.
- Triapitsy S., Mizell RF, Bossart JL, Carlton CE . 1998. Egg parasitoids of *Homalodisca coagulata* (Homoptera: Cicadellidae). *Florida Entomologist*. 81: 241-243.

#### **FUNDING AGENCIES**

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# THE ALIMENTARY TRACK OF GLASSY-WINGED SHARPSHOOTER AS A TARGET FOR CONTROL OF PIERCE'S DISEASE, AND DEVELOPMENT OF MIMETIC INSECTICIDAL PEPTIDES FOR GLASSY-WINGED SHARPSHOOTER CONTROL

## Project Leader:

Brian A. Federici  
Dept. of Entomology  
University of California  
Riverside, CA 92521

**Reporting Period:** The results reported here are from work conducted from December 2, 2003 to October 15, 2004.

## ABSTRACT

Transgenic insecticidal crops expressing *Bacillus thuringiensis* (*Bt*) toxins have been successfully developed to control major chewing insect pests of agriculture, such as caterpillars and beetles. The same *Bt* toxin technology also has been used with *Bacillus sphaericus* for the control of mosquito species such as *Aedes aegypti* and *Culex quinquefasciatus*, important vectors of human diseases. However, this transgenic technology has not yet been applied to economically important xylem-feeding sucking insect pests such as the glassy-winged sharpshooter, *Homalodisca coagulata* (GWSS). Our goal is to use a genomics approach to develop novel, highly specific mimetic insecticidal proteins derived from the variable binding domains of immunoglobulin molecules. "Mimetic" peptides mimic the normal substrates of key components of essential processes to block the activities of these proteins. Our research is targeting the exposed active domains of transport proteins on the surface of the GWSS midgut microvillar membrane and enzymes found in GWSS saliva. Degenerate PCR amplification of genes characterized in other insect species encoding proteins involved in gut transport and saliva activity and screening a cDNA microarray to identify novel gut and saliva protein encoding genes are the approaches being used to identify GWSS target proteins. Due to the target specificity, mimetic peptide technology can provide an environmentally sound approach to the control of vasculature feeding insect pests and could thereby provide a means of controlling Pierce's disease and crop losses due to GWSS feeding.

## INTRODUCTION

Mimetic technology is new to agriculture, but has been used extensively and successfully in medicine (Clemens, 1996). Examples of medical uses include the inactivation of disease-related enzymes (Burke et al., 2001), blockage of metabolic receptors important to disease (Berezov et al., 2000), and the use of antibodies developed against disease constituents (Moe et al., 1999). Human cancers (Monzayi-Karbassi and Keiber-Emmons, 2001), diabetes (Deghenghi, 1998), and heart disease (Lincoff et al., 2000) all have been treated successfully through these applications of mimetic technology. In spite of lacking a history of application of mimetics to agriculture problems, its development should be straight forward. Antibody proteins have been synthesized successfully in plants for the production of antibodies to be used in medical applications (Larrick et al. 2001; Stoger et al., 2002), and the production of transformed lines of crop plants in which promoters that have been isolated by other researchers (Shi et al., 1994; Springer, 2000), which direct expression to the cell wall and vascular structures of plants, will assure that our antibody peptides are synthesized in a tissue-specific manner. Last year we succeeded in isolating portions of five GWSS genes by degenerate PCR: the A and c V-ATPase subunits, genes encoding trypsin-like and maltase-like saliva proteins, and a membrane transporter. This year we have added another membrane transporter gene clone, most closely related to the potassium coupled amino acid transporter isolated from *Manduca sexta*, KAAT1 (Castagna et al., 1998). These clones and others isolated from our normalized cDNA are being analyzed using bioinformatics tools to identify functional domains which will be effective and specific targets. The identified target peptides will be synthesized in a *Baculovirus* expression system. Peptides produced will be used as antigens for polyclonal antibody production, the products of which will be cloned into phage display libraries. Screening the phage display antibody libraries will identify the mimetic peptides that bind most efficiently to the targeted GWSS proteins. Ultimately these peptides will be used in feeding studies to identify those which are the best candidates for GWSS control.

## OBJECTIVES

1. Determine the structure and cell types in the midgut epithelium and salivary glands of the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata*;
2. Prepare a normalized cDNA microarray of GWSS using pooled cDNAs isolated from each developmental stage.
3. Screen the microarray using cDNA probes derived from midgut and salivary gland tissue-specific probes to determine the tissue-specific expression of key midgut microvillar and saliva proteins;
4. Clone and sequence genes encoding one or more key midgut microvillar and saliva proteins and determine their suitability as targets for a molecular biological approach to GWSS and Pierce's disease control.
5. Predict functional domains of key GWSS midgut epithelium- and salivary gland-specific proteins based on sequences of genes using bioinformatics;
6. Express functional domain peptides for antibody production;
7. Clone single-chain fragment variable antibody genes into recombinant phage libraries and screen the libraries;
8. Conduct feeding studies to identify efficacious mimetic peptides effective in killing or deterring GWSS.

## RESULTS

We have had a normalized cDNA library constructed by Evrogen JSC from total RNA isolated from whole GWSS of both sexes and all life stages, as well as from GWSS that have fed on grape infected with *X. fastidiosa*. We've had 10,752 clones isolated, glycerol stocks prepared, and PCR products of all inserts amplified and purified for microarray spotting. This August three members of our laboratory were trained at the Custom Microarray Facility at the University of Arizona and we are currently repeating the results obtained there at the Core Instrumentation Facility in the Institute for Integrative Genome Biology on the Riverside campus. A subset of 1,536 clones was spotted in duplicate (side by side spots) and the entire array duplicated on the same slide. These arrays were hybridized to Cy3 labeled control cDNA and Cy5 labeled cDNA reverse transcribed and amplified from total RNA isolated from GWSS treated with a sub-lethal dose or an LD50 dose of esfenvalerate. Dye swap experiments were performed. These experiments are part of a collaborative related project funded by CDFA with Frank Byrne as Project Leader. Our results are presented in his report for the project entitled "Evaluation of resistance potential in the glassy-winged sharpshooter (GWSS) using toxicological, biochemical and genomics approaches." The arrays detected obvious differences in gene expression levels between the two treatments. These experiments were chosen for our test study because it is known that several genes encoding cytochrome P450 proteins are up-regulated dramatically in response to pesticide treatment. We have succeeded in cloning the entire GWSS V-ATPase A gene (Figure 1) by RLM-RACE. Differences in both the 5'- and 3'- sequences between the clones obtained indicate more than one copy of the V-ATPase A gene exists in the GWSS genome

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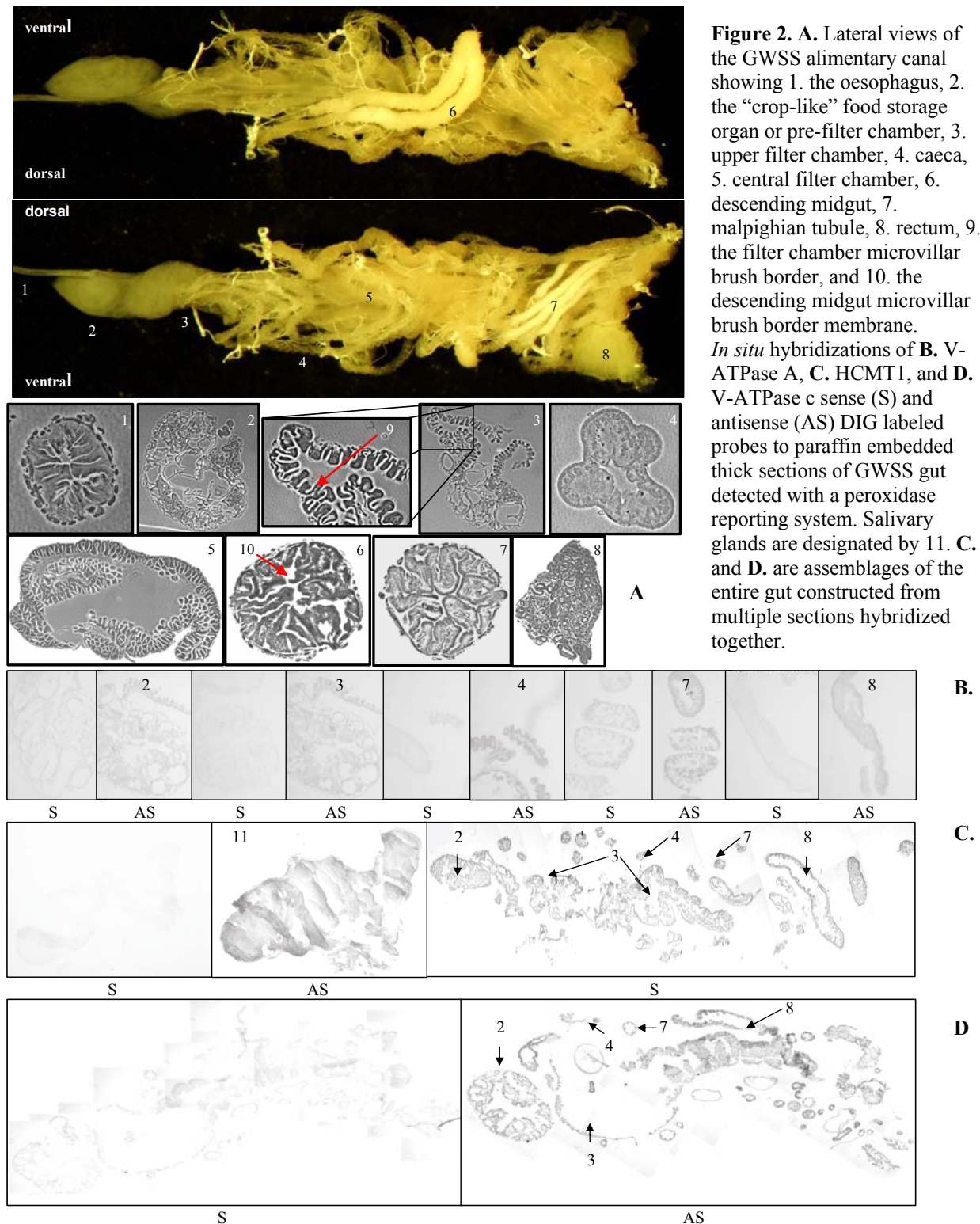
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               M S
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594  atcgcatcccctggcaactacactgttgatgatgtgttcttctgaaacggagtttgacgggtgagaagtctaagtatacaatgttgcaagtggtgacctga
   I A S P G N Y T V D D V V L E T E F D G E K S K Y T M L Q V W P V
693  cgtcagcccagacctgtgaccgagaagctgccagccaatcaccactgtctcactgggtcaacgtgtgctcgactctctattcccttgtgtgcaaggtggt
   R Q P R P V T E K L P A N H P L L T G Q R V L D S L F P C V Q G G
792  accacggccatccctggagccttcggttgcggtgaaactgtcatctctcaggccctgtccaaataactccaactcggatgtcatcatttacgtaggatgt
   T T A I P G A F G C G K T V I S Q A L S K Y S N S D V I I Y V G C
891  ggagagcgaggaaatgaaatgtctgaggtattgcaagacttccctgagctgtctgtagagattgatggagtcacggagagtataatgaagcgaactgtc
   G E R G N E M S E V L Q D F P E L S V E I D G V T E S I M K R T A
990  ctggtagccaacacctccaacatgcctgtcgccgcccagagaagcttccatttatacaggaatcacactgtctgaaatatttccgagacatgggtctacaat
   L V A N T S N M P V A A R E A S I Y T G I T L S E Y F R D M G Y N
1089  gtttctatgatggctgactctacatcccggttgggcccgaagccctgagagaaatttctggacgattggcagaaatgctgtgacagtggttaccgccga
   V S M M A D S T S R W A E A L R E I S G R L A E M P A D S G Y P A
1188  tacctgggtgcccgtctagcctccttctacgagagagccggctcgagtcaagtgcctgggaaatcccgatcgtgaaggctctgtcagttattgtcggtgtc
   Y L G C P S S L L L R X S R S S Q V P G N P D R E G S V S I V G A
1287  gtgtcacctcctggtggtgacttctcagatcccgctcacctccgccacccttgggtatcgtacaggtcttctggggtcttgacaagaaattggcacaaaagg
   V S P P G G D F S D P V T S A T L G I V Q V F W G L D K K L A Q R
1386  aaacactttccctccattaactggctcatatcatacagtaatacatgagagcactagatgacttctatgacaagagcttcccagagtttgtacccttg
   K H F P S I N W L I S Y S K Y M R A L D D F Y D K S F P E F V P L
1485  aggaccaaggtaaaggagattctgcaggaggaagaagatttgcagaaattgtacaactggttggcaaagcatcacttggcgaactgacaaaatcacc
               gagtcc
               S
   R T K V K E I L Q E E E D L S E I V Q L V G K A S L A E T D K I T
1584  ctcgaggtcaataggctactgaaagaagattttctgcaacagaacagctactctccgtatgatcgtttctgtcccttctacaagacagtggtgcatgctc
   L E V A R L L K E D F L Q Q N S Y S P Y D R F C P F Y K T V G M L
1683  cgcaacatgattgcattctttgacatgtcaaggcatgcccgtcgagtcgacagcacaaagtgaataacagatcacctggagtgcatcaaggacggcatg
   R N M I A F D M S R H A V E S T A C G S E N K I T W S V I K D G M
1782  ggcaacattctgtaccaactgtcgtcaatgaaattcaaggatcccgtgaaagatgaaaaaaaaa
   G N I L Y Q L S S M K F K D P V K D E

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**Figure 1.** The complete cDNA and translated protein of GWSS V-ATPase A. The atg indicates the translational start site. Nucleic acid and protein sequence variations are indicated in bold. Sequence variations were determined from both sense and antisense sequences.



We have dissected and identified all of the components of the GWSS alimentary canal, performed ultrastructural studies of these tissues, and developed *in situ* hybridization techniques for the localization of gene expression (Figure 2). As expected the genes encoding the V-ATPase A and c subunits and that expressing HcMT1 are all expressed throughout the GWSS gut. HcMT1 clearly also is expressed in the salivary glands. The studies localizing the expression of the trypsin-like and maltase-like genes are in progress.



Transcript sizes for each of the genes partially cloned have been determined by RNA blot hybridization (Figure 3). The transcript sizes were determined as: ~1,900 bp for V-ATPase A, which corresponds well with that determined from the cDNA sequence of 1,849 bp, ~1,200bp for V-ATPase c, and ~875 bp for HcMT1 and the trypsin-like gene. These values



correspond to those in the literature for each of these genes (van Hille *et al.* 1993; Pietrantonio and Gill, 1993; Zeng *et al.*, 2002; Liu *et al.*, unpublished data).

## CONCLUSIONS

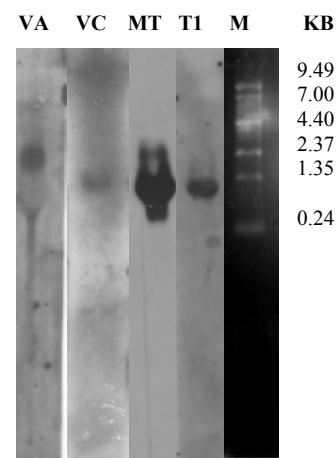
The presence of more than one GWSS V-ATPase A subunit gene will be confirmed by DNA blot hybridization. We have developed a clone capture technique which will allow us to isolate all gene clones with sequence similarity from our cDNA library in a single experiment. This procedure involves the formation of a RecA-mediated triple-stranded molecule between our biotinylated partial clone and full length cDNA clones with sequence similarity. Triple-stranded molecules are then removed from the reaction using streptavidin magnetic beads. This approach will allow us to much more quickly analyze all the members of specific gene families already partially cloned. Thus far we have succeeded in isolating clones similar to the KAAT-like gene clone recently obtained (data presented in the report of a related project: Development of Glassy-winged Sharpshooter Mimetic Insecticidal Peptides, and an Endophytic Bacterial System For Their Delivery to Mature Grape.). The clones isolated are being analyzed to identify the regions best suited for antibody targeting using bioinformatics tools. We anticipate that this approach also will allow us to isolate gene families of genes identified by microarray screening as being tissue-specifically expressed. This will be important in determining that a potential target does not have similarity to genes expressed other than in the organs we want to target.

## REFERENCES

- Berezov, A., H-T. Zhang, M. Greene, and R. Murali. 2001. Disabling ErbB receptors with rationally designed exocyclic mimetics of antibodies: structure-function analysis. *J. Med. Chem.* 44: 2565-2574.
- Burke, T., Z-J. Yao, D-G. Liu, J. Voigt, and Y. Gao. 2001. Phosphoryltyrosyl mimetics in the design of peptide-based signal transduction inhibitors. *Biopolymers.* 60: 32-44.
- Castagna, M., C. Shayakul, D. Trotti, V. Sacchi, W. Harvey, and M. Hediger. 1998. Cloning and characterization of a potassium-coupled acid transporter. *Proc. Natl. Acad. Sci. USA.* 95: 5395-5400
- Clemens, U. 1996. Current concepts of treatment in medical oncology: New anticancer drugs. *J. of Cancer Res. Clin. Oncol.* 122: 189-198.
- Deghenghi, R. 1998. Synthetic peptides and their non-peptidyl mimetics in endocrinology: From synthesis to clinical perspectives. *J. Endocrin. Invest.* 21: 787-793.
- Larrick, J., L. Yu, C. Naftzger, S. Jaiswal, and K. Wycoff. 2001. Production of secretory IgA antibodies in plants. *Biomolecular Engineering.* 18: 87-94.
- Lincoff, A., R. Califf, and E. Topol. 2000. Platelet glycoprotein IIb/IIIa receptor blockade in coronary artery disease. *J. Amer. Coll. Card.* 35: 1103-1115.
- Liu, J., J. Zhang, R. Zhou, and C. Jin. Molecular cloning and preliminary study on biological function of a novel gene overexpressed in human hepatocellular carcinoma. unpublished . accession number CAB81951.1
- Moe, G., S. Tan, and D. Granoff. 1999. Molecular mimetics of polysaccharide epitopes as vaccine candidates for prevention of *Neisseria meningitidis* serogroup B disease. *FEMS Immun. Med. Micro.* 26: 209-226.
- Monzayi-Karbassi, B., and T. Kieber-Emmons. 2001. Current concepts in cancer vaccine strategies. *Biotechniques.* 30: 170-189.
- Pietrantonio, P.V., and S.S. Gill. 1993. Sequence of a 17 kDa vacuolar H(+)-ATPase proteolipid subunit from insect midgut and Malpighian tubules. *Insect Biochem Mol Biol.* 6: 675-680.
- Shi, Y., M. Wang, K. Powell, E. Van Damme, V. Hilder, A. Gatehouse, D. Boulter, and J. Gatehouse. 1994. Use of the rice sucrose synthase-1 promotor to direct phloem-specific expression of  $\beta$ -glucuronidase and snowdrop lectin genes in transgenic tobacco plants. *J. Exp. Botany.* 45: 623-631.
- Springer, P. 2000. Gene traps: Tools for plant development and genomics. *Plant Cell.* 12: 1007-1020.
- Stoger, E., M. Sack, R. Fischer, and P. Christou. 2002. Plantibodies: Applications, advantages and bottlenecks. *Curr. Opinion Biotech.* 13: 161-166.
- Van Hille B., H. Richener, D.B. Evans, J.R. Green, and G. Bilbe. 1993. Identification of two subunit A isoforms of the vacuolar H(+)-ATPase in human osteoclastoma. *J Biol. Chem.* 268: 7075-7080
- Zeng F., Y.C. Zhu, A.C. Cohen . 2002. Molecular cloning and partial characterization of a trypsin-like protein in salivary glands of *Lygus hesperus* (Hemiptera: Miridae). *Insect Biochem. Mol. Biol.* 4: 455-464.

## FUNDING AGENCIES

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**Figure 3.** RNA blot hybridizations of 10 $\mu$ g GWSS total RNA hybridized to V-ATPase A (VA), V-ATPase c (VC) and the trypsin-like gene (T1) clones labeled with DIG and detected with chemiluminescence and the HCMT1 (MT) gene clone labeled with 32P.

# REALIZED LIFETIME PARASITISM AND THE INFLUENCE OF BROCHOSOMES ON FIELD PARASITISM RATES OF GLASSY-WINGED SHARPSHOOTER EGG MASSES BY *GONATOCERUS ASHMEADI*

## Project Leaders:

Mark Hoddle  
Dept. of Entomology  
University of California  
Riverside, CA 92521

## Cooperators:

Robert Luck	Nic Irvin
Dept. of Entomology	Dept. of Entomology
University of California	University of California
Riverside, CA 92521	Riverside, CA 92521

**Reporting period:** The results reported here are from work conducted from July 2004 to October 2004.

## ABSTRACT

### INTRODUCTION

GWSS is an exotic pest in California having invaded and established in this state in the late 1980's. One potential reason for the inordinate numbers of GWSS in California compared to population densities in the pest's home range in southeastern USA is a lack of an efficient natural enemy fauna that has evolved to use GWSS as a resource. As part of a classical biological control program against GWSS, scientists with the CDFA and UCR have been prospecting for, importing into quarantine, and clearing for release mymarid egg parasitoids from the home range of GWSS for establishment in California. To date, two new parasitoid species have been established in CA, *Gonatocerus triguttatus* and *G. fasciatus*. It is too early to ascertain the impact on GWSS population growth that these two parasitoids will have. The self introduced *G. ashmeadi* (Vickerman et al., 2004) is the key natural enemy of GWSS egg masses in CA at present (Blua et al., 1999). Over summer, parasitism levels of GWSS egg masses and individual eggs in masses by *G. ashmeadi* approaches 100% but parasitism levels of the spring generation of GWSS are substantially lower (Triapitsyn and Phillips, 2000). Naturally occurring populations of *G. ashmeadi* in CA have been augmented with mass reared individuals from populations found in the southeastern USA and northeastern Mexico which encompasses the home range of GWSS (D. Morgan - CDFA, pers. comm. 2003).

Substantial laboratory work with *G. ashmeadi* has been conducted in an attempt to understand and parameterize basic aspects of this parasitoid's reproductive biology, and host selection behaviors. Irvin and Hoddle (2001) have evaluated oviposition preferences of *G. ashmeadi* when presented GWSS eggs of various ages. Interspecific competition between *G. ashmeadi* with *G. triguttatus* and *G. fasciatus* for GWSS egg masses of different ages has been assessed along with factors influencing the sex ratio of offspring (Hoddle and Irvin, 2002; 2003). The effect of resource provisioning and nutrient procurement on the longevity of *G. ashmeadi* has also been determined (Irvin unpublished data). Furthermore, the foraging efficacy of *G. ashmeadi* in simple and complex environments for scarce and abundant GWSS egg masses has also been completed and compared to similar data collected for *G. triguttatus* (Irvin unpublished data).

The effect of brochosomes on the foraging efficacy of *G. ashmeadi* has also been evaluated in the laboratory. Brochosomes are a chalky material produced by the malpighian tubules in many xylophagous cicadellid species (Rakitov, 1999; 2000; 2004). Brochosomes are excreted from specialized openings on the posterior of the abdomen and are collected and deposited by mated females on the forewings. During oviposition, females rub brochosomes off the forewings and deposit them on the tops of eggs masses (Hix, 2001). The adaptive significance of covering egg masses with brochosomes is uncertain (Rakitov, 1999). Hix (2001) has suggested that brochosomes may protect GWSS eggs from desiccation, UV light, natural enemies (parasitoids, predators and pathogens); or they provide a signal to other female GWSS that leaves have already been oviposited in. We have investigated the effect of brochosomes on the foraging efficacy of *G. ashmeadi* in the laboratory. Data clearly demonstrate that moderate to heavy brochosome coverage of GWSS eggs is a major impediment to oviposition to *G. ashmeadi* when compared to conspecific parasitization efficiency of GWSS eggs with light or no brochosome coverage (Velema et al., 2004).

Studies currently funded by the CDFA to be conducted by this lab will look at: (1) laboratory-level fecundity rates of *G. ashmeadi* under varying temperature regimens; (2) field cage studies assessing interspecific competition between parasitoids released for the classical biological control of GWSS; (3) factors affecting sex ratio allocation during mass production of mymarid parasitoids; and (4) the effect of resource provisioning on parasitization rates and overwintering longevity of key mymarid parasitoids under field conditions. The work proposed in this grant will complement and support completed studies and work in progress.

Many factors act in concert to affect successful biological control. The GWSS-*Gonatocerus* system has benefited from intensive laboratory study to generate a basic understanding of factors influencing host selection and parasitism success. The

next step that is now required is to test hypotheses generated from lab studies in the field. Field level assessments will evaluate our understanding of the system under investigation, and consolidate interpretations needed to determine the most important aspect of the GWSS biological control program: “How effective are egg parasitoids at controlling GWSS in California?” To get to the crux of this issue we are asking two questions in this proposal: (1) How big an impact do individual female parasitoids have on GWSS population growth via parasitization of eggs, and (2) do biotic impediments such as brochosomes affect parasitization efficacy in the field? When these two questions are addressed together we will begin to develop a comprehensive understanding of the impacts parasitoids have at the field level and factors affecting parasitization success. This will allow us to form a much better understanding of what levels of control we can expect from mymarid egg parasitoids when different ecological conditions are prevailing in the field.

## OBJECTIVES

This is a new proposal that was officially funded in July 2004. This project has two objectives aimed at determining the field level impact individual female *Gonatocerus ashmeadi* have on glassy-winged sharpshooter (GWSS) egg masses. These two research objectives are complimentary:

1. Measure real life time contributions of individual female parasitoids to parasitism of GWSS egg masses under field conditions. This research objective is high priority.
2. Determine the ecological significance of brochosome deposition on GWSS egg masses and its effect on parasitism rates by *G. ashmeadi* under field conditions.

## RESULTS

This project has not commenced. There are two major reasons for this: (1) Recruitment of Dr. Nic Irvin as the post-graduate researcher for this program has been held up by the excessive time it has taken to process the required visas to employ her in the USA given her alien status. (2) Dr. Irvin will start working on this project in early March 2005 when GWSS populations begin to build again. It made no sense to employ Dr. Irvin earlier than this time as at the time of notification of successful visa application GWSS populations were declining in the field and there would be few reproductive adults and parasitoids to work with. We will be formally requesting a no cost extension for this project.

## REFERENCES

- Blua, M.J., Phillips, P.A., and Redak, R.A. 1999. A new sharpshooter threatens both crops and ornamentals. *Calif. Agric.* 53: 22-25.
- Irvin, N. and Hoddle, M.S. 2001. Egg age preference and “window of susceptibility” of *Homalodisca coagulata* eggs to attack by *Gonatocerus ashmeadi* and *G. trigtutatus*. Pierce’s Disease Control Program. Symposium Proceedings, Pierce’s Disease Research Symposium, December 5-7, 2001. Coronado Island Marriott Resort. pp. 135-136.
- Hix, R.L. 2001. Egg-laying and brochosome production observed in glassy-winged sharpshooter. *Calif. Agric.* 55: 19-22.
- Hoddle M.S. and Irvin, N. 2002. Interspecific competition between *Gonatocerus ashmeadi* and *G. trigtutatus* for glassy-winged sharpshooter egg masses. Pierce’s Disease Control Program. Symposium Proceedings, Pierce’s Disease Research Symposium, December 15-18, 2002. Coronado Island Marriott Resort. pp. 86-87.
- Hoddle, M.S. and Irvin, N. 2003. Interspecific competition between *Gonatocerus ashmeadi*, *G. trigtutatus*, and *G. fasciatus* for glassy-winged sharpshooter egg masses. Pierce’s Disease Control Program. Symposium Proceedings, Pierce’s Disease Research Symposium, December 8-11, 2003. Coronado Island Marriott Resort. pp. 250-254.
- Rakitov, R.A. 1999. Secretory products of the malpighian tubules of Cicadellidae (Hemiptera: Membracoidea): an ultrastructural study. *International Journal of Insect Morphol. Embryol.* 28: 179-193.
- Rakitov, R.A. 2000. Secretion of brochosomes during the ontogenesis of a leafhopper. *Oncometopia orbona* (F.) (Insecta, Homoptera, Cicadellidae). *Tissue & Cell* 32: 28-39.
- Rakitov, R.A. 2004. Powdering of egg nests with brochosomes and related sexual dimorphism in leafhoppers (Hemiptera: Cicadellidae). *Zool. J. Linn. Soc.* 104: 353-381.
- Triapitsyn, S.V. and Phillips, P.A. 2000. First record of *Gonatocerus trigtutatus* (Hymenoptera: Mymaridae) from eggs of *Homalodisca coagulata* (Homoptera: Cicadellidae) with notes on the distribution of the host. *Florida Entomologist* 83: 200-203.
- Velema H.P., Hoddle, M.S. Hemerik, L. and Luck, R.F. 2004. The influence of brochosomes on parasitisation efficiency of *Gonatocerus ashmeadi* (Girault) (Hymenoptera: Mymaridae), parasitising *Homalodisca coagulata* (Say) (Hemiptera: Cicadellidae) egg-masses. *Ecol. Entom. Submitted*
- Vickerman, D., Hoddle, M.S., Triapitsyn S. and Stouthamer, R. 2004. Species identity of geographically distinct populations of the glassy-winged sharpshooter parasitoid *Gonatocerus ashmeadi*: Morphology, DNA sequences and reproductive compatibility. *Biol. Cont. In Press.*

## FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce’s Disease and Glassy-winged Sharpshooter Board.

# REPRODUCTIVE AND DEVELOPMENTAL BIOLOGY OF *GONATOCERUS ASHMEADI*, AN EGG PARASITOID OF THE GLASSY-WINGED SHARPSHOOTER

**Project leader:**

Mark Hoddle  
Dept. of Entomology  
University of California  
Riverside, CA 92521

**Cooperator:**

Leigh Pilkington  
Dept. of Entomology  
University of California  
Riverside, CA 92521

**Reporting period:** The results reported here are from work conducted from April 2004 to October 2004.

**ABSTRACT**

The reproductive and developmental biology of *Gonatocerus ashmeadi* Girault, a self-introduced parasitoid of the glassy-winged sharpshooter (GWSS) *Homalodisca coagulata* Say, was determined at five constant temperatures in the laboratory; 15; 20; 25; 30; and 33°C. Wasps at each experimental temperature were given, on average, between 10 and 15 GWSS eggs per day for its natural life for oviposition. At 30°C, immature *G. ashmeadi* sustained the highest mortality rates as adult emergence was lowest at this temperature. The largest proportion of female offspring was produced at 25°C and lifetime fecundity was greatest at 25°C. The development time was greatest at 15°C and lowest at 30°C. Mean adult longevity was inversely related to temperature with a maximum of approximately 30 days at 15°C to a minimum of approximately two days at 33°C.

**INTRODUCTION**

The mymarid wasp species *Gonatocerus ashmeadi* Girault, *G. triguttatus* Girault, *G. morrilli* Howard, and *G. fasciatus* Girault are the most common natural enemies associated with the insect pest *Homalodisca coagulata* in its home range of southeastern USA and northeastern Mexico (Triapitsyn and Phillips, 2000). The wasp *G. ashmeadi* is a self-introduced resident of California and most likely came into the state in parasitized *Homalodisca coagulata* eggs (Vickerman et al., 2004) and has established widely in association with *H. coagulata*.

One factor that can limit the success of the establishment of natural enemies is mismatching the environmental conditions favored by the introduced agent with those that predominate in the receiving range (Hoddle, 2004). Quantification of the reproductive and developmental biology of a natural enemy is paramount to predicting, planning, and promoting the establishment and population growth of introduced agents. This can be enhanced by determining demographic characteristics such as day-degree requirements for immature development, population doubling times and lifetime fecundity for estimating population growth rates at various temperatures and for comparison with the target pest and other species of biological control agents. Determining the introduced control agent's reproductive and developmental biology and environmental requirements with that of the host will allow for a greater understanding of factors affecting biological control of GWSS.

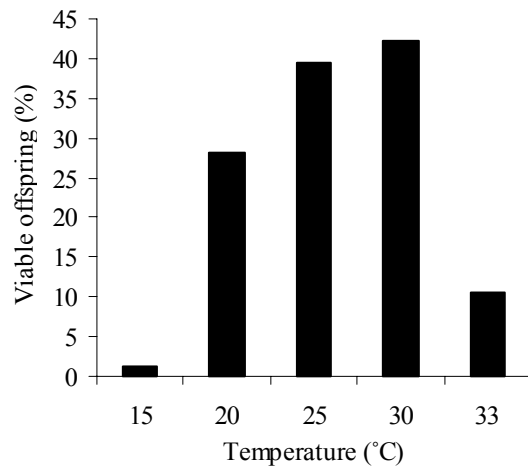
The following work was undertaken to provide information on the reproductive and developmental biology of the parasitoid wasp *G. ashmeadi*. These data will provide knowledge of the insect's life cycle, in particular in relation to GWSS, and will improve the understanding of optimal timings of its release for biological control purposes in many agricultural systems as well as improve the efficiency of laboratory rearing of these insects. In addition to improving release and rearing strategies, this information will target foreign exploration of strains of *G. ashmeadi* for possible introduction into California and also identify geographical areas that will be conducive to the use of this species as biological control agent following GWSS establishment in various parts of California and in areas such as Tahiti and Hawaii where GWSS has recently invaded.

**OBJECTIVES**

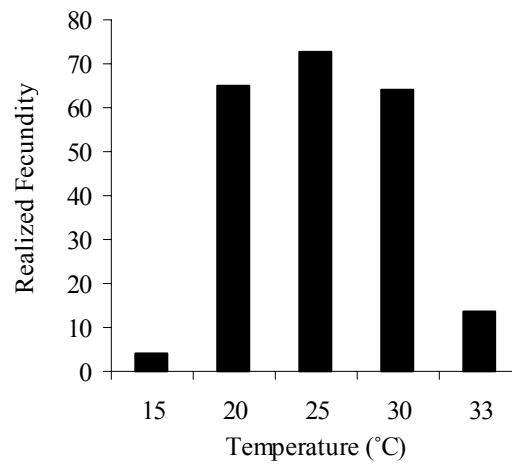
1. Examine the developmental and reproductive biology of *G. ashmeadi* in order to determine its day-degree requirements, and demographic statistics.

**RESULTS**

The rates for oviposition that led to successful reproduction of offspring were highest at 30°C (Figure 1). Each wasp at each temperature, on average, had the same number of GWSS eggs made available to them for oviposition. At 30°C, approximately 42% of eggs presented to wasps produced into viable parasitoid offspring. Conversely, this rate decreased with temperature to 1% at 15°C. Higher temperatures similarly lowered the production of viable offspring with approximately 13% surviving to adult stages at 33°C. These results suggest that *G. ashmeadi* progeny survivorship was most successful when oviposition occurred at 30°C, intermediate at 20-25°C and lowest at 15°C.



**Figure 1.** Mortality rates fell as temperatures rose until 30°C. Few viable offspring were produced at 33°C. The highest percentage of viable offspring from available eggs was at 30°C.

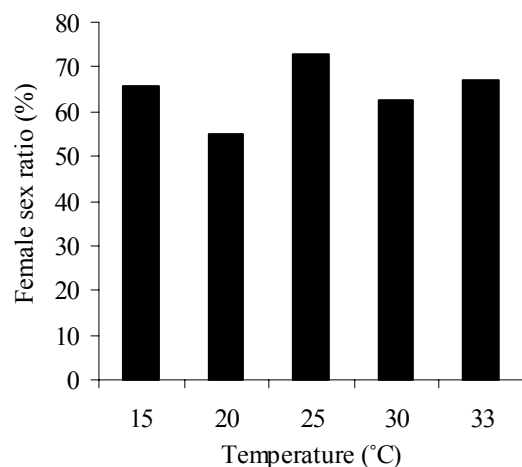


**Figure 2.** The average number of offspring emerging from parasitized eggs at each temperature. Parasitized eggs that did not yield viable offspring are not represented here.

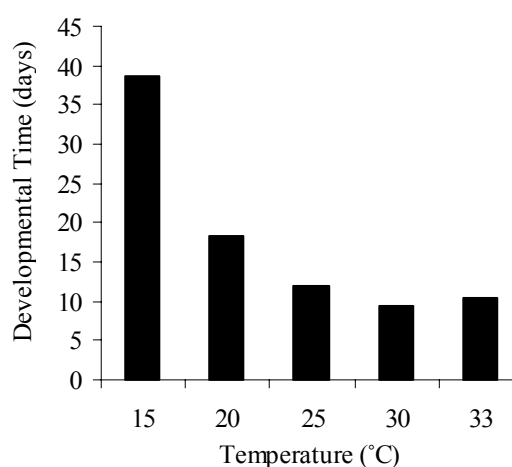
The number of offspring produced by individual wasps over their lifetime was greatest at 25°C and fell sharply as temperature either increased or decreased (Figure 2). Approximately 73 offspring were produced by wasps at 25°C down to an average of around 4 and 14 at 15°C and 33°C, respectively. These data show that at constant high or low temperatures wasps fail to produce many offspring and may have little or no impact on GWSS population growth as a consequence.

There appeared to be no trends to the ratios of females produced at each experimental temperature (Figure 3). The highest percentage of females was produced at 25°C with approximately 70% of offspring being female. All other temperatures were, with the exception of 20°C, within 10% of this temperature. These results indicate that temperature may not play an important role in the sex selection of *G. ashmeadi* offspring.

The time between eggs being made available to individual wasps and the emergence of offspring, fell from a high of approximately 39 days at 15°C to approximately 10 days for 30 – 33°C (Figure 4). As temperature rose, the time required for the development of wasp larvae was reduced. This faster development time at higher temperatures suggests that wasps will cycle through several generations in comparison to GWSS.

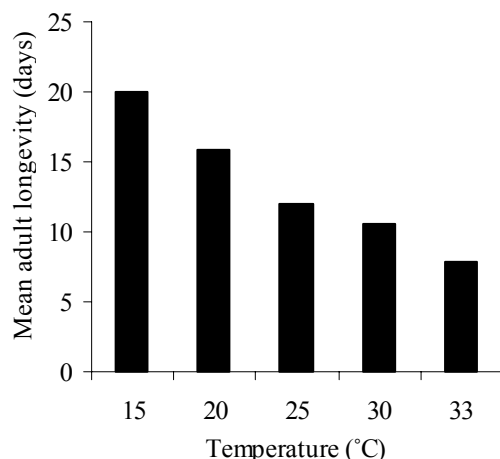


**Figure 3.** The percentage of *G. ashmeadi* offspring that was identified as female at each temperature.



**Figure 4.** The period of time between oviposition by *G. ashmeadi* and the emergence of wasp offspring represented in days.

Mean adult longevity for individual mated female *G. ashmeadi* used in this study fell from an average of approximately 20 days at 15°C to approximately eight days at 33°C (Figure 5).



**Figure 5.** The average time, in days, from when mated females used in the study first emerged to when they died of natural causes.

## CONCLUSIONS

The wasps at 30°C died quicker (figure 5) and laid fewer eggs (figure 3) than wasps at 25°C. This difference was offset by the findings that the individuals at 30°C successfully utilized a higher percentage of the eggs that were made available to them than those at 25°C. Whilst individuals at 30°C produced fewer viable offspring, it is possible that as a population effect greater numbers of offspring may be produced due to a faster generation turnover and higher rate of parasitism overall. Wasps at 30°C will cause a population to grow at a much faster rate due to the wasp ovipositing in, largely, an equal number of eggs. The success of the wasp at this temperature is indicative of the much shorter developmental times whereby the wasp will produce offspring that develop at much faster rates. Individual wasps surviving for extended periods of time were observed at 15°C and declining in a linear manner as temperature rose. Whilst wasps at 15°C, for example, survived considerably longer than at other temperatures their efficacy was affected by the temperature and made very little impact on the number of offspring produced.

The success of a biological control agent is measured by the mortality it inflicts on its target which is in part a function of its reproductive and developmental activity across a range of temperatures (Nahrung and Murphy, 2002). The results from this study suggest that *G. ashmeadi* operates most effectively at moderate to high temperatures. Identifying the optimal temperature for reproduction and development of *G. ashmeadi*, will greatly aid mass-rearing efforts, using day-degree models to predict geographic range, to assess generational turnover in various locales in comparison to GWSS and to optimize releases of natural enemies into a field environment.

## REFERENCES

- Hoddle M.S., 2004. The potential adventive geographic range of glassy-winged sharpshooter, *Homalodisca coagulata* and the grape pathogen *Xylella fastidiosa*: Implications for California and other grape growing regions of the world. *Crop Protect.* 23, 691-699.
- Nahrung H.F., Murphy, B.D., 2002. Differences in egg parasitism of *Chrysophtharta agricola* (Chapuis) (Coleoptera : Chrysomelidae) by *Enoggera nassau* Girault (Hymenoptera : Pteromalidae) in relation to host and parasitoid origin. *Austral. J. Entomol.* 41, 267-271.
- Triapitsyn S.V., Phillips, P.A., 2000. First record of *Gonatocerus triguttatus* (Hymenoptera : Mymaridae) from eggs of *Homalodisca coagulata* (Homoptera : Cicadellidae) with notes on the distribution of the host. *Florida Entomologist* 83, 200-203.
- Vickerman D.B., Hoddle, M.S., Triapitsyn, S.V., Stouthamer, R., 2004. Species identity of geographically distinct populations of the glassy-winged sharpshooter parasitoid *Gonatocerus ashmeadi*: Morphology, DNA sequences and reproductive compatibility. *Biol. Cont. In Press.*

## FUNDING AGENCIES

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# SEARCHING FOR AND COLLECTING EGG PARASITOIDS OF THE GLASSY-WINGED SHARPSHOOTER AND OTHER *HOMALODISCA* SPECIES IN SOUTHEASTERN AND SOUTHWESTERN MEXICO

## Project Leaders:

Mark S. Hoddle  
Dept. of Entomology  
University of California  
Riverside, CA 92521

Serguei V. Triapitsyn  
Dept. of Entomology  
University of California  
Riverside, CA 92521

## Cooperators:

David J. W. Morgan  
California Dept. of Food and Agriculture  
Riverside, CA 92521

Svetlana Myartseva  
Universidad Autónoma de Tamaulipas  
Cd. Victoria, Tamaulipas, Mexico

**Reporting Period:** The results reported here are from work conducted from July 1, 2004 to October 6, 2004.

## ABSTRACT

According to the proposed (and approved) research timetable, work on this project will commence as early as in January 2005, when we may have the first chance to collect parasitized egg masses of *Homalodisca* spp. in Mexico. This report is only for information purposes about this project.

## INTRODUCTION

Egg parasitoids of the Glassy-winged Sharpshooter (GWSS), *Homalodisca coagulata* (Say), were discovered through survey activities conducted throughout USA and northeastern Mexican states of Nuevo Leon and Tamaulipas, which resulted in collection, introduction, and release in California of several species of mymarid eggs parasitoids (*Gonatocerus* spp.) (Morgan et al. 2000; Triapitsyn et al., 2002; Triapitsyn & Hoddle, 2001, 2002). During 2003 and 2004, we conducted a survey of egg parasitoids of GWSS in central and eastern USA (Hoddle & Triapitsyn, 2003, 2004). According to McKamey (2002), the native host range of GWSS also includes central and southern Mexico, well beyond the currently known range mapped by Triapitsyn & Phillips (2000). McKamey's (2002) report is supported by the CLIMEX-predicted distribution range of GWSS (Hoddle 2004; also Map below).

Here we propose the final step in the development of a classical biological control program against GWSS in California: to search new climatically suitable areas in Mexico for GWSS parasitoids. Additionally, our previous exploratory work in Mexico (in the States of San Luis Potosí, Tamaulipas and Veracruz) during 1999-2003 resulted in the discovery of at least two new, undescribed species of *Homalodisca* egg parasitoids, which are related to *G. ashmeadi* Girault and *G. morrilli* (Howard) but differ from those both morphologically (Triapitsyn et al. 2002) and genetically (D. Vickerman, unpubl. data). These parasitoids need to be recollected in Mexico and tested as potential biological control agents against GWSS in California.

## OBJECTIVES

This project has two main objectives:

1. Search for and collect egg parasitoids in southern-most home range of GWSS and other *Homalodisca* species in southeastern and southwestern Mexico; and
2. Introduction and establishment of quarantine cultures of the selected species and their following initial evaluation for potential establishment in California.

## RESULTS

There are no results to be reported at this time. The following experimental procedures will be used to accomplish the above objectives:

### *Exploratory Work.*

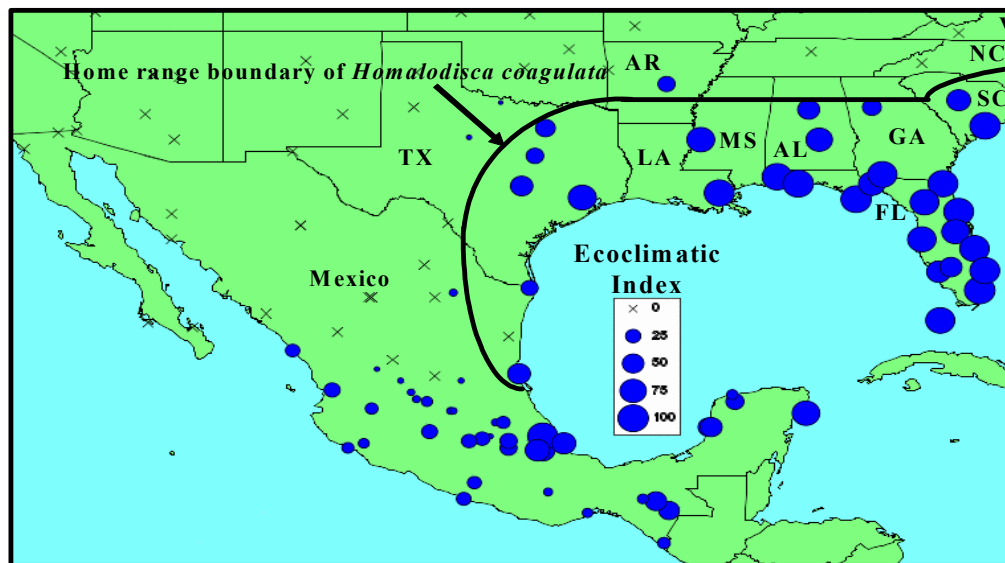
Search for and collect egg parasitoids of southern-most home range of GWSS and other *Homalodisca* species (in the Mexican states of Tamaulipas (southern part), Veracruz, San Luis Potosí, Campeche, Oaxaca, Yucatán, and Quintana Roo) for introduction into California, establishment of cultures in UCR quarantine, and a following evaluation. Several short exploratory trips will be made to those states during winter and spring 2005 and parasitized egg masses of *Homalodisca* will be collected there and sent to UCR quarantine facility under the existing permit. The two already known egg parasitoids of GWSS from Tamaulipas and adjacent Mexican states (*G. near ashmeadi* and *G. near morrilli*) will be recollected from known localities.

### *Quarantine and Identification Work*

Colonies of the selected parasitoids will be established in UCR quarantine using GWSS as a host (fresh egg masses will be supplied by David Morgan). Voucher specimens of the collected parasitoids will require appropriate curation as a result of

the proposed exploratory work; these will need to be critically point-dried from ethanol, point- or card-mounted, labeled, slide-mounted, and identified to genera and species. DNA analyses will be conducted if necessary.

**Figure 1.** Current and CLIMEX-predicted geographical range of GWSS. Large blue dots indicate good climatic conditions for GWSS. Small blue dots are marginal habitats. x on map indicate unsuitable areas.



## CONCLUSIONS

Research to be conducted in the course of this project will be of benefit primarily to the CDFA GWSS Biological Control Program as well as to other biocontrol specialists and agencies conducting projects against GWSS in California such as the USDA-APHIS. Ultimately, we hope that this project will be beneficial to California's agriculture.

## REFERENCES

- Hoddle, M. S. 2004. The potential adventive geographic range of glassy-winged sharpshooter, *Homalodisca coagulata*, and the grape pathogen *Xylella fastidiosa*: implications for California and other grape growing regions of the world. *Crop Protect. In press*.
- Hoddle, M. S. and S. V. Triapitsyn. 2003. Searching for and collecting egg parasitoids of the Glassy-winged Sharpshooter in the central and eastern USA, pp. 261-262. *In: Proceedings of the Pierce's Disease Research Symposium, December 8-11, 2003, Coronado Island Marriott Resort, Coronado, California. Organized by California Department of Food and Agriculture (compiled by M. Athar Tariq, S. Oswalt, P. Blincoe, R. Spencer, L. Houser, A. Ba and T. Esser), Copeland Printing, Sacramento, California, 323 p.*
- Hoddle, M. S. and S. V. Triapitsyn. 2004. Searching for and collecting egg parasitoids of the Glassy-winged Sharpshooter in the central and eastern USA. *In: Proceedings of the Pierce's Disease Research Symposium, December 7-10, 2004.*
- McKamey, S. H. 2002. Developing a stable classification of the glassy-winged sharpshooter genus *Homalodisca*, pp. 122-123. *In: Proceedings of the Pierce's Disease Research Symposium, December 15-18, 2002, Coronado Island Marriott Resort, Coronado, California. Organized by California Department of Food and Agriculture (compiled by M. Athar Tariq, S. Oswalt, and T. Esser), Digital Logistix, Sacramento.*
- Morgan, D. J. W., S. V. Triapitsyn, R. A. Redak, L. G. Bezark, and M. S. Hoddle. 2000. Biological control of the glassy-winged sharpshooter: current status and future potential, pp.167-171. *In: [Proceedings] California Conference on Biological Control, held July 11-12, 2000 at the historic Mission Inn, Riverside, California (M. S. Hoddle, Ed.), 205 pp.*
- Triapitsyn, S. V., L. G. Bezark and D. J. W. Morgan. 2002. Redescription of *Gonatocerus atriclavus* Girault (Hymenoptera: Mymaridae), with notes on other egg parasitoids of sharpshooters (Homoptera: Cicadellidae: Proconiini) in northeastern Mexico. *Pan-Pacific Entomologist* 78: 34-42.
- Triapitsyn, S. V. and M. S. Hoddle. 2001. Search for and collect egg parasitoids of glassy-winged sharpshooter in southeastern USA and northeastern Mexico, pp. 133-134. *In: Proceedings of the Pierce's Disease Research Symposium, December 5-7, 2001, Coronado Island Marriott Resort, San Diego, California. Organized by California Department of Food and Agriculture (compiled by M. Athar Tariq, S. Oswalt & T. Esser), Copeland Printing, Sacramento, California, 141 pp.*



- Triapitsyn, S. V. and M. S. Hoddle. 2002. Search for and collect egg parasitoids of glassy-winged sharpshooter in southeastern USA and northeastern Mexico, pp. 94-95. In: Proceedings of the Pierce's Disease Research Symposium, December 15-18, 2002, Coronado Island Marriott Resort, San Diego, California. Organized by California Department of Food and Agriculture (compiled by M. Athar Tariq, S. Oswalt, P. Blincoe and T. Esser), Digital Logistix, Sacramento, California, 177 p.
- Triapitsyn, S. V. and P. A. Phillips. 2000. First host record of *Gonatocerus triguttatus* (Hymenoptera: Mymaridae) from eggs of *Homalodisca coagulata* (Homoptera: Cicadellidae), with notes on the distribution of the host. Florida Entomologist 83: 200-203.

#### **FUNDING AGENCIES**

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# SEARCHING FOR AND COLLECTING EGG PARASITOIDS OF GLASSY-WINGED SHARPSHOOTER IN THE CENTRAL AND EASTERN USA

## Project Leaders:

Mark S. Hoddle  
Dept. of Entomology  
University of California  
Riverside, CA 92521

Serguei V. Triapitsyn  
Dept. of Entomology  
University of California  
Riverside, CA 92521

## Cooperators:

Roman A. Rakitov  
Illinois Natural History Survey  
Champaign, IL

David J. W. Morgan  
California Dept. of Food and Agriculture  
Riverside, CA

**Reporting Period:** The results reported here are from work conducted from January 1, 2004 to October 6, 2004.

## ABSTRACT

Search for egg parasitoids of proconiine sharpshooters (Hemiptera: Clypeorrhyncha: Cicadellidae: Cicadellinae: Proconiini) in central and eastern USA during 2003 and 2004 resulted in rearings of several species of Mymaridae and Trichogrammatidae (Hymenoptera) (Table 1). Cultures of some species, notably of *Anagrus epos* Girault, were established at UCR quarantine.

## INTRODUCTION

Presence of the proconiine sharpshooters *Homalodisca coagulata* (Say) (GWSS - the Glassy-winged Sharpshooter) and its close relative *Oncometopia orbona* (Fabricius) (the Broad-headed Sharpshooter) in central and eastern United States justified conducting a survey of their principal natural enemies, egg parasitoids in the families Mymaridae and Trichogrammatidae. No such surveys have ever been conducted North of central Georgia, Mississippi, Louisiana, and Texas. Prior research showed presence of the mymarid *Gonatocerus fasciatus* Girault there (Triapitsyn et al. 2003). A number of trichogrammatid genera and species were recognized in southeastern USA from eggs of a grass-inhabiting *Cuerna costalis* (Fabricius), also a proconiine sharpshooter, as well as from *H. coagulata* and *O. orbona* (Triapitsyn 2003).

## OBJECTIVES

1. **Exploratory work** - Search for and collect egg parasitoids of proconiine sharpshooters in the northern- and eastern-most home range of GWSS, *Oncometopia* spp., and *Cuerna* spp. for introduction into California, establishment of cultures in UCR quarantine, and a following evaluation.
2. **Curatorial work** – Curate the collected voucher specimens of mymarid and trichogrammatid egg parasitoids.

## RESULTS

### *Objective 1.*

The first exploratory trip was made to Kentucky and Tennessee by S. Triapitsyn in July 2003 (Hoddle & Triapitsyn 2003). The second trip to Illinois (the northernmost distribution range of *Oncometopia orbona* and *Cuerna costalis*), eastern Kentucky, and south-central Tennessee was made by S. Triapitsyn in April 2004, in an attempt to locate and collect the overwintered and egg-laying adults of *C. costalis*. Part of the trip (in southern Illinois) was made together with Roman Rakitov, who showed his methods of collecting *C. costalis* in known localities where this species had been collected in the past (occurrence of proconiine sharpshooters there is spotty). We were able to collect several adults of *C. costalis* in one locality in Shawnee National Forest, on a private meadow. Yellow pan traps were placed in this locality and we managed to collect a specimen of *Gonatocerus novifasciatus* Girault (Mymaridae), a known parasitoid of *H. coagulata* elsewhere. There it most probably is parasitoid of *Cuerna costalis*, the only proconiine sharpshooter occurring on that meadow. This gave us a hint what species of egg parasitoids occur there despite the fact that it is practically impossible to find egg masses of this proconiine sharpshooter when its density is so low. Also parasitoids and leafhoppers were collected there using vacuum. In several locations in southern Illinois, both methods revealed frequent presence of *Gonatocerus rivalis* Girault and its likely host, *Draculacephala antica* (Walker) (determined by Roman Rakitov). *Draculacephala* is a cicadelline (tribe Cicadellini) sharpshooter genus, which members were the most abundant leafhoppers of the subfamily Cicadellinae in all three states visited. This could be an apparent new host association for this species of *Gonatocerus*, which is a member of the *sulphuripes* species group.

Subsequent trips to Georgia, North Carolina, and South Carolina in June and August 2004 by S. Triapitsyn resulted in collections of several mymarid and trichogrammatid species, listed in Table 1, which were reared from egg masses of proconiine sharpshooters. Quarantine colonies of *Gonatocerus ashmeadi* Girault from Georgia and South Carolina were discontinued several generations following their establishment because it was shown that this species is morphologically, biologically, and genetically homogenic throughout its range (Vickerman et al. 2004). Both GWSS and to some degree *O. orbona* were found to be abundant almost everywhere in the lowlands (especially coastal) in Georgia, North Carolina, and

South Carolina whereas GWSS could not be found in the forested hills and mountains of northern Georgia, eastern North Carolina, Kentucky, and Tennessee, where only a few adult *O. orbona* as well as its old egg masses (all with evidence of parasitization) were collected.

Our survey also benefited greatly from the exploratory work by Roman Rakitov, who reared mymarid and trichogrammatid egg parasitoids of several species of the genus *Cuerna* (other than *C. costalis*). Particularly, the mymarid *Anagrus epos* Girault was reared by Roman Rakitov near Glyndon, Clay County, Minnesota, from egg masses of a *Cuerna* sp. and sent to UCR quarantine facility under a permit. This is the first representative of the genus *Anagrus* ever reared from eggs of a proconiine sharpshooter. We were able to establish a quarantine colony of this species on eggs of GWSS, which is a fictitious host for *A. epos* (GWSS does not occur in Minnesota). *Anagrus epos* is a gregarious species: 3-5 adult wasps emerged from smaller eggs of the original host, *Cuerna* sp., whereas up to 10-12 adult wasps emerged from larger eggs of GWSS. Under quarantine laboratory conditions (temperature 24°C, RH ca. 50%), the first two generations of *A. epos* developed from egg to adult within 20-21 days; for unknown reasons, it took the next two generations much longer (more than 30 days) to develop under the same conditions. Currently, this species is under quarantine evaluation as a potential biocontrol agent against GWSS in California.

**Table 1.** Species of egg parasitoids collected during 2004 and sent to University of California, Riverside quarantine.

Genus and species of egg parasitoid	Originally from: (State: locality)	Original or probable sharpshooter host	Propagated on GWSS at UCR quarantine (Yes/No)
<i>Acmopolynema sema</i> Schauff (Mymaridae)	GA: nr. Centerville	? <i>Homalodisca insolita</i> (Walker)	No
<i>Gonatocerus ashmeadi</i> Girault (Mymaridae)	GA: nr. Centerville	<i>H. coagulata</i> / <i>O. orbona</i>	No
	GA: Byron	<i>H. coagulata</i> / <i>O. orbona</i>	Yes
	NC: Garner	<i>H. coagulata</i>	No
	NC: North Myrtle Beach	? <i>H. coagulata</i>	No
	NC: nr. Warsaw	<i>H. coagulata</i>	No
	SC: Charleston	<i>H. coagulata</i>	Yes
<i>Gonatocerus fasciatus</i> Girault (Mymaridae)	SC: nr. Yemassee	<i>H. coagulata</i> / <i>O. orbona</i>	Yes
	GA: nr. Centerville	<i>H. coagulata</i> / <i>O. orbona</i>	No
	GA: Byron	<i>H. coagulata</i> / <i>O. orbona</i>	No
	NC: Garner	<i>H. coagulata</i>	No
	NC: nr. Greensboro	? <i>O. orbona</i>	No
	NC: nr. Warsaw	<i>H. coagulata</i>	No
<i>Zagella spirita</i> (Girault) (Trichogrammatidae)	GA: Byron	<i>H. coagulata</i> / <i>O. orbona</i>	No (failed)
<i>Ufens</i> new species (Trichogrammatidae)	GA: Byron	<i>H. coagulata</i> / <i>O. orbona</i>	No (failed)
<i>Paracentrobia acuminata</i> (Ashmead) (Trichogrammatidae)	GA: nr. Centerville	? <i>H. insolita</i> / ? <i>Cuerna costalis</i>	No

## Objective 2

As a result of the exploratory work conducted during the reported period, numerous specimens of proconiine sharpshooters and of their egg parasitoids were collected and preserved in ethanol with appropriate labels; many of these were critically point-dried from ethanol, point- or card-mounted, labeled, and identified to genera and species. Representatives of some species (of both sexes) were selected, dissected, and slide-mounted. The specimens were deposited in the collections of Entomology Research Museum, UC Riverside.

## CONCLUSIONS

This is the next step in the development of a “classical” biological control program for the reduction of glassy-winged sharpshooter (GWSS) densities in California as a cornerstone for an IPM program to manage GWSS. As the result of our surveys conducted during 1997-2004, several previously unknown proconiine sharpshooter host associations were discovered for various species of Mymaridae and Trichogrammatidae. We concluded searching for egg parasitoids of GWSS in the Nearctic part of its distribution range. Next year, our exploratory efforts will focus on the southernmost part of the distribution range of GWSS in southern Mexico, which is in the Neotropical region.

## REFERENCES

- Hoddle, M. S. and S. V. Triapitsyn. 2003. Searching for and collecting egg parasitoids of the Glassy-winged Sharpshooter in the central and eastern USA, pp. 261-262. In: Proceedings of the Pierce's Disease Research Symposium, December 8-11, 2003, Coronado Island Marriott Resort, Coronado, California. Organized by California Department of Food and Agriculture (compiled by M. Athar Tariq, S. Oswalt, P. Blincoe, R. Spencer, L. Houser, A. Ba & T. Esser), Copeland Printing, Sacramento, California, 323 p.
- Triapitsyn, S. V. 2003. Taxonomic notes on the genera and species of Trichogrammatidae (Hymenoptera) - egg parasitoids of the proconiine sharpshooters (Hemiptera: Clypeorrhyncha: Cicadellidae: Proconiini) in southeastern USA. Trans. Amer. Entomol. Soc. 129: 245-265.
- Triapitsyn, S. V., D. J. W. Morgan, M. S. Hoddle and V. V. Berezovskiy. 2003. Observations on the biology of *Gonatocerus fasciatus* Girault (Hymenoptera: Mymaridae), egg parasitoid of *Homalodisca coagulata* (Say) and *Oncometopia orbona* (Fabricius) (Hemiptera: Clypeorrhyncha: Cicadellidae). Pan-Pacific Entomologist 79 (1): 75-76.
- Vickerman, D. B., M. S. Hoddle, S. Triapitsyn, and R. Stouthamer. 2004. Species identity of geographically distinct populations of the glassy-winged sharpshooter parasitoid *Gonatocerus ashmeadi*: morphology, DNA sequences, and reproductive compatibility. Biol. Cont. 31: 338-345.

## FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

## DEVELOPMENT OF PEPTIDE ANTIBIOTIC-BASED CONTROL STRATEGIES FOR *XYLELLA FASTIDIOSA*

### Project Leaders:

Shizuo George Kamita  
Dept. of Entomology  
University of California  
Davis, CA 95616

Bruce D. Hammock  
Dept. of Entomology  
University of California  
Davis, CA 95616

### Cooperators:

Bruce Kirkpatrick  
Dept. of Plant Pathology  
University of California  
Davis, CA 95616

Donald S. Warkentin  
Dept. of Entomology  
University of California  
Davis, CA 95616

**Reporting Period:** The results reported here are from work conducted from July 2003 to September 2004.

### ABSTRACT

Peptide antibiotics are short (generally less than 70 amino acid residue-long), pore forming peptides encoded by single genes. Because peptide antibiotics are ‘gene-based’ they can be produced directly at the target location where they are needed (e.g., grape stock). In this project, we are testing the hypothesis that peptide antibiotics such as cecropins A, B, and/or P1 can be used as an effective means to control or reduce the spread of *Xylella fastidiosa*-induced disease. During the reporting period, we have established the optimal growth and assay conditions for the *X. fastidiosa* bacterium. Under these optimal conditions, we found that cecropin A can effectively inhibit *X. fastidiosa* growth over a two-week period (initial concentration of 0.05 $\mu$ M). Longer-term growth inhibition was seen only when higher concentrations of cecropin A were used suggesting that the cecropin A is being degraded under the conditions of our assay. On the basis of the effectiveness of cecropin A against *X. fastidiosa*, a synthetic plant codon (i.e., *Arabidopsis thaliana*) optimized, cecropin A gene was synthesized. The product of this synthetic cecropin A gene was expressed using the baculovirus expression vector system (BEVS) in insect cells. In insect cells roughly 90 mg/liter of culture of biologically active cecropin A was produced by the recombinant baculovirus. Following confirmation of biological activity of the insect cell produced cecropin A, the synthetic cecropin A gene was inserted into the pCambia1305 series of plasmid vectors in order to express the cecropin A in transgenic *A. thaliana* and eventually grape stock. Four different recombinant pCambia1305 vectors were generated (carrying either the pro or mature cecropin A gene fused to either an authentic insect- or plant- (rice glycine-rich protein) derived signal peptide sequence). We are currently in the process of generating transgenic *A. thaliana* using these pCambia vectors. We believe that continuous expression (although potentially at relatively low levels) of cecropin A will be effective for reducing or inhibiting the growth of *X. fastidiosa* within the plant.

### INTRODUCTION

Traditional antibiotics are natural or chemically synthesized small molecules that can selectively kill or stop the growth of bacteria. Antibiotic inhibition of *X. fastidiosa* (at least 17 isolates tested) has been analyzed for six different antibiotics (ampicillin, kanamycin, neomycin, penicillin, streptomycin, and tetracycline) [1, 2]. These studies demonstrate that antibiotic treatment is potentially an effective method for the control of *X. fastidiosa*. Under field conditions, however, barriers between the antibiotic and bacterium, and degradation effects will require significantly higher application doses than those found effective in the laboratory. Such doses may be impractical especially for broad-spectrum antibiotics due to secondary effects (e.g., toxicity against mammalian red blood cells) and the risk of increasing resistance. Thus, although traditional antibiotics such as tetracycline are highly active, an effective delivery system to bring them in contact with *X. fastidiosa* in the plant or insect vector is not available.

Recently, a great deal of scientific effort is being put into the study of a second type of antimicrobial agent called peptide antibiotics. Peptide antibiotics have been identified from a wide range of organisms including bacteria, fungi, plants, insects, birds, crustaceans, amphibians and mammals. In general, peptide antibiotics are small (less than 50 amino acids), have a net positive charge, and are composed of 50% or more of hydrophobic amino acids [3, 4]. One class of peptide antibiotic is composed of so-called ribosomally synthesized peptides [5]. These peptides are encoded by single genes and synthesized by a protein complex (ribosome) that is found in all cells and processed following synthesis via common pathways [3, 6]. In other words, unlike traditional antibiotics, peptide antibiotics have the potential to be easily produced by common protein expression systems or in transgenic organisms (e.g., plants). Furthermore, because peptide antibiotics are “gene-based”, they can be produced directly at the location where they are needed and their synthesis can potentially be regulated by using appropriate gene promoters.

Some of the best-characterized peptide antibiotics are the cecropins. Cecropins were the first peptide antibiotics to be identified in an animal, the giant silkworm *Hyalophora cecropia* [7, 8]. At least ten different cecropins have been isolated from lepidopteran (moths and butterflies) and dipteran (flies) insects [9, 10]. Cecropins are composed of a single chain of 35-39 common L-amino acids and do not contain disulfide bonds [10]. Cecropins are active against many Gram(-) bacteria and

some Gram(+) bacteria, but are inactive against eukaryotic cells at concentrations that are antimicrobial [4, 9, 11] and possibly at concentrations up to 300 times higher [8]. *X. fastidiosa* is a Gram(-) bacterium [12]. In Gram(-) bacteria, the antibacterial activities of cecropins A, B, and P1 are up to ten-times greater than tetracycline [9, 13]. Cecropins have a unique combination of characteristics (specificity, gene basis, small size, potency against Gram(-) bacteria, etc.) that may make them potentially ideal substances for the control of *X. fastidiosa* in GWSS.

## OBJECTIVES

- I. Identify peptide antibiotics (cecropins) that are effective against *Xylella fastidiosa*
  - i. Determine the antibiotic sensitivity of *X. fastidiosa* to chemically synthesized cecropins
  - ii. Produce recombinant cecropins using baculovirus expression vectors
  - iii. Determine the toxicity of cecropins against GWSS cells grown in culture
- II. Analyze the effectiveness of cecropins produced in transgenic *Arabidopsis*
  - i. Generate transgenic *Arabidopsis* expressing cecropin that is active against *X. fastidiosa*
  - ii. Determine the localization, yield, activity, and stability of plant-expressed cecropin
  - iii. Analyze the effect of cecropin expression on the transgenic *Arabidopsis*
  - iv. Analyze the effectiveness of plant-expressed cecropin for the control of *X. fastidiosa* transmission

## RESULTS AND CONCLUSIONS

In order to establish the optimal conditions for the growth, storage, and assay of *X. fastidiosa* (Temecula strain) in our laboratory, we tested three different media (PD3, PW, and GYE; see [14]) and various inoculation routines. In general, our procedures were modified from protocols established in the Bruce Kirkpatrick laboratory at U.C. Davis. Optimal conditions for the generation of bacterial (*X. fastidiosa*) lawns for agar disc diffusion assays were also determined. Of the three media that were tested, PD3 gave the fastest growth of *X. fastidiosa* in liquid medium (roughly 20- and 135-fold increases in the OD<sub>600</sub> at 7 and 14 days post inoculation, respectively) and on agar plates (formation of a lawn by 7-10 days post seeding). In order to generate a lawn, 150 µL of a 14 day-old culture (OD<sub>600</sub>=0.48-0.5) was spread onto a 10 cm-diameter plate containing PD3 agar medium.

Using the optimal growth conditions with PD3 medium, we examined the minimal inhibitory concentration (MIC assay) at which cecropins A, B, and P1 (commercially purified peptides) were effective in inhibiting the growth of *X. fastidiosa*. We found that cecropins A, B, and P1 were effective at partially inhibiting the growth of *X. fastidiosa* at concentrations that were equal to or greater than 0.05, 0.25, and 0.5 µM, respectively, at two weeks post inoculation (Table 1). In general, cecropin A was the most effective against *X. fastidiosa*. The effectiveness of the cecropins as well as kanamycin was reduced by three weeks post inoculation. This was speculated to be the result of antibiotic degradation.

Once the sensitivity of *X. fastidiosa* to the various cecropins was established, a codon-optimized (for *A. thaliana*) cecropin A gene (pro gene including the insect-derived signal peptide sequence) was synthesized using commercially synthesized oligomers. A comparison of the *A. thaliana*-optimized (upper) and authentic (lower) cecropin A gene sequences is as follows:

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ATGAACCTCTCTAGAACTCTTCTTCTCGTGTTCGCTTGCCTCACTGCTCTCGCTATGGTGAACGCTGCTCCTGAGCCTAAGTGAAGCTCTTCAAGAAGA 100
ATGAACCTTTTCGAGGATCTTTTCTTCTCGTGTTCGCTTGCCTGACGCTCTAGCAATGGTCAATGCGCGCCGGAACCTAAATGGAAGTTATTCAAGAAGA 100
***** ** * ***** ***** ***** ** ***** ** ***** ** ***** ** ***** ** ***** ** ***** ** ***** **
TGCAGAAAGTGGGTGAGAACATCAGAGATGGAATCATCAAGGCTGGACCAGCTGTGGCTGTGGTGGGACAGGCTACACAGATCGCTAAGGGTTGA 195
TTGAGAAAGTCGGTCAGAACATTCGAGATGGCATCATCAAGCTGGCCAGCCGTCGCTGTTGTAGGCCAGGCAACACAGATTGCTAAGGGTTAA 195
** ***** ***** ***** ***** ** ***** ** ***** ***** ***** ***** ***** ***** ***** **

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Of the 195 nucleotides that encode the pro gene, 33 nucleotides were mutated for optimal expression in *A. thaliana* (and putatively in grape stock). The synthesized gene was directionally cloned into the baculovirus transfer vector pAcUW21 at the *Bgl*III and *Eco*RI sites. Subsequently, the recombinant transfer vector was used to generate a recombinant baculovirus (vAcCecA) expressing the cecropin A gene using standard procedures. Expression of biologically active cecropin A was confirmed by minimal inhibitory concentration assays using *E. coli* by comparison of vAcCecA- or wildtype AcMNPV-infected insect Sf-21 cell culture supernatants or cell extracts (Table 2). These experiments confirmed that the synthetic gene encoded a functional peptide and that this peptide was correctly processed in insect-derived cells. vAcCecA expressed high levels (roughly 90 mg/liter of insect cell culture (2 x 10<sup>6</sup> cells/mL)) of cecropin A.

Following confirmation that the synthetic gene produces biologically active cecropin A, the synthetic gene was inserted into the pCAMBIA1305 series of plasmid vectors in order to express the cecropin A in transgenic *A. thaliana* (and eventually grape stock). Four different recombinant pCAMBIA1305 vectors were generated by PCR-amplification as follows:

1. pro cecropin A sequence with authentic insect signal peptide sequence
2. pro cecropin A sequence with rice glycine rich protein and authentic insect signal peptide sequences
3. mature cecropin A sequence with rice glycine rich protein signal peptide sequence

#### 4. mature cecropin A with no signal peptide sequence

The authenticity of the PCR-amplified sequences was confirmed by nucleotide sequencing in both directions and the constructs are currently being used to generate transgenic *A. thaliana* by standard procedures.

**Table 1.** Effect of cecropins and kanamycin against the growth of *X. fastidiosa*

	Concentration ( $\mu$ M)	Increase in bacterial concentration in comparison to cultures lacking antibiotic		
		Week 1 (% $\pm$ s.d.)	Week 2 (% $\pm$ s.d.)	Week 3 (% $\pm$ s.d.)
cecropin A	0.5	69 $\pm$ 3	47 $\pm$ 47	64 $\pm$ 42
	0.25	72 $\pm$ 10	80 $\pm$ 21	117 $\pm$ 5
	0.1	103 $\pm$ 13	68 $\pm$ 2	87 $\pm$ 25
	0.05	110 $\pm$ 46	50 $\pm$ 1	91 $\pm$ 22
cecropin B	0.5	69 $\pm$ nd	114 $\pm$ 6	87 $\pm$ 45
	0.25	63 $\pm$ 31	75 $\pm$ nd	110 $\pm$ 15
	0.1	72 $\pm$ 101	128 $\pm$ 63	90 $\pm$ nd
	0.05	93 $\pm$ 17	101 $\pm$ 18	74 $\pm$ 10
cecropin P1	0.5	98 $\pm$ 18	70 $\pm$ 40	70 $\pm$ 62
	0.25	82 $\pm$ 18	98 $\pm$ nd	120 $\pm$ 17
	0.1	111 $\pm$ 52	93 $\pm$ 24	72 $\pm$ 24
	0.05	93 $\pm$ 10	99 $\pm$ 22	73 $\pm$ 18
kanamycin	2	11 $\pm$ 3	9 $\pm$ 8	16 $\pm$ 2
	1	19 $\pm$ 8	32 $\pm$ 39	33 $\pm$ 22
	0.5	42 $\pm$ 16	77 $\pm$ 9	103 $\pm$ 16
	0.25	60 $\pm$ 13	72 $\pm$ 17	105 $\pm$ 12
nd = not determined				

**Table 2.** Effect of recombinant cecropin A on the growth of *E. coli*

Source of recombinant cecropin A	Inoculum dose (bacteria/mL)	Inhibition (%)
Sf21 cell pellet ( $1 \times 10^5$ cells)	$1.1 \times 10^3$	3.1 $\pm$ 13.2
Sf21 cell supernatant (undiluted)	$1.1 \times 10^3$	99.7 $\pm$ 0.1
Sf21 cell supernatant (undiluted)	$1.0 \times 10^4$	57.9 $\pm$ 1.6
Sf21 cell supernatant (undiluted)	$8.5 \times 10^4$	51.6 $\pm$ 0.2
Sf21 cell supernatant (undiluted)	$7.3 \times 10^5$	13.1 $\pm$ 0.1
Sf21 cell supernatant (1:5 diluted)	$7.0 \times 10^5$	11.1 $\pm$ 0.2
Sf21 cell supernatant (1:10 diluted)	$7.0 \times 10^5$	2.5 $\pm$ 0.1

#### REFERENCES

1. Darjean, D.H., E.L. Civerolo, and B.C. Kirkpatrick, *In vitro* growth inhibition of *Xylella fastidiosa* by selected metallic plant micronutrients and antibiotics. *Phytopathology*, 2000. **90**(6 Supplement): p. S17-S18.
2. Lacava, P.T., et al., *RAPD* profile and antibiotic susceptibility of *Xylella fastidiosa*, causal agent of citrus variegated chlorosis. *Letters in Applied Microbiology*, 2001. **33**(4): p. 302-306.
3. Hancock, R.E.W. and G. Diamond, *The role of cationic antimicrobial peptides in innate host defences*. *Trends in Microbiology*, 2000. **8**(9): p. 402-410.
4. Tossi, A., L. Sandri, and A. Giangaspero, *Amphipathic, alpha-helical antimicrobial peptides*. *Biopolymers*, 2000. **55**(1): p. 4-30.
5. Hancock, R.E.W. and D.S. Chapple, *Peptide antibiotics*. *Antimicrobial Agents and Chemotherapy*, 1999. **43**(6): p. 1317-1323.
6. Mor, A., *Peptide-based antibiotics: A potential answer to raging antimicrobial resistance*. *Drug Development Research*, 2000. **50**(3-4): p. 440-447.

7. Hultmark, D., et al., *Insect Immunity: Purification and properties of three inducible bacterial proteins from hemolymph of immunized pupae of Hyalophora cecropia*. European Journal of Biochemistry, 1980. **106**: p. 7-16.
8. Steiner, H., et al., *Sequence and specificity of two antibacterial proteins involved in insect immunity*. Nature (London), 1981. **292**(16): p. 246-248.
9. Boman, H.G., *Cecropins: antibacterial peptides from insects and pigs*, in *Phylogenetic Perspectives in Immunity*, C. Janeway, Editor. 1994, Lanes Biomed.: Austin.
10. McDonald, C., *Cecropins: A Class of Lytic Peptides*. 1997, Pacific West Cancer Fund: Seattle. p. 1-24.
11. Hancock, R.E.W. and R. Lehrer, *Cationic peptides: A new source of antibiotics*. Trends in Biotechnology, 1998. **16**(2): p. 82-88.
12. Henderson, M., et al., *Genetic diversity of Pierce's disease strains and other pathotypes of Xylella fastidiosa*. Applied and Environmental Microbiology, 2001. **67**(2): p. 895-903.
13. Boman, H.G., *Peptide antibiotics and their role in innate immunity*. 1995. p. 61-92.
14. Campanharo, J.C., M.V.F. Lemos, and E.G.D. Lemos, *Growth optimization procedures for the phytopathogen Xylella fastidiosa*. Current Microbiology, 2003. **46**(2): p. 99-102.

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## MICROBIAL CONTROL OF THE GLASSY-WINGED SHARPSHOOTER WITH ENTOMOPATHOGENIC FUNGI

### Project Leader:

Harry K. Kaya  
Dept. of Nematology  
University of California  
Davis, CA 95616

### Researcher:

Surendra K. Dara  
Shafter Research and Extension Center  
University of California-Davis  
Shafter, CA 93263

### Cooperator:

Michael R. McGuire  
USDA, ARS  
Shafter Research and Extension Center  
Shafter, CA 93263

**Reporting Period:** The results reported here are from work conducted from April 2004 to September 2004.

### ABSTRACT

Objectives of our study were to search for fungal pathogens of the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata* (Say) and evaluate their potential against the host. Searches within citrus orchards in Tulare and Riverside counties revealed no natural infections of entomopathogenic fungi in GWSS populations. Entomopathogenic fungi were also absent in cadavers of GWSS periodically collected from Riverside citrus orchards (courtesy CDFA) when incubated in the laboratory under ideal conditions for fungal emergence. However, about 140 isolates of *Beauveria bassiana* (Balsamo) Vuillemin and four isolates of *Metarhizium anisopliae* (Metschnikoff) Sorokin, both hyphomycetous fungi, were isolated from soil in GWSS habitats and other insect hosts. Some of these isolates along with a Weslaco isolate of *B. bassiana* from GWSS and a commercial *B. bassiana* isolate have been tested against GWSS. Preliminary results indicate that GWSS is susceptible to high concentrations of these fungi.

### INTRODUCTION

The glassy-winged sharpshooter (GWSS), *Homalodisca coagulata* (Say), native to the southeastern United States, is a serious pest of the California grape industry because it vectors *Xylella fastidiosa* (Wells et al. 1987), a xylem-limited bacterium that causes Pierce's disease (PD). Although PD has been in California for a long time, the introduction and rapid spread of GWSS made the situation worse. In addition to grapes, GWSS has a wide host range and spreads various diseases in those hosts caused by *X. fastidiosa*. Vector control or avoidance has been a key tactic in controlling PD. Widely practiced chemical control with imidacloprid and application of kaolin particles have their limitations. While kaolin particles, although non-toxic, can leave unwanted deposits on the harvested grape bunches, chemical insecticides have undesirable effects including human health, impact on non-target organisms, and environmental concerns. Moreover, use of chemical insecticides in citrus disrupts the successful, long-term control afforded by IPM of many different citrus pests (Grafton-Cardwell and Kallsen 2001). Use of microbial agents, such as entomopathogenic fungi, can be a viable alternative that is compatible with IPM practices. Entomopathogenic fungi invade the host by penetrating through the integument and are appropriate candidates for GWSS that has piercing and sucking mouthparts.

Entomopathogenic fungi have been isolated from GWSS (Mizell and Boucias 2002, Jones - personal communication) and other cicadellids (Galaini-Wraight et al. 1991, Hywel-Jones et al. 1997, Magalhaes et al. 1991, Matsui et al. 1998, McGuire et al. 1987). The purpose of our study is to discover additional isolates of entomopathogenic fungi active against GWSS.

### OBJECTIVES

1. Conduct surveys to find fungal infections in GWSS populations or insects closely related to GWSS and isolate soilborne entomopathogens from GWSS habitats.
2. Culture and isolate the fungi and evaluate their pathogenicity against GWSS.
3. Evaluate the host range of fungi that infect GWSS.
4. Conduct small-scale field tests to evaluate selected pathogens against GWSS on citrus in fall and winter.

### RESULTS

#### *Natural Infections in GWSS Populations*

Citrus orchards in Tulare and Riverside counties were surveyed, in vain, for infected GWSS. GWSS cadavers from CDFA collections in the Riverside area were periodically obtained and incubated in the laboratory for fungal development. No entomopathogenic fungus has so far been found from these cadavers. However, cultures of *Beauveria bassiana* (Balsamo) Vuillemin from infected GWSS collected in Texas by Jones and *Hirsutella* spp collected in Florida by Mizell and Boucias were received in the past two months for testing against California GWSS.

### Isolation of Fungal Pathogens

Soil samples were collected from an organic citrus orchard and a conventional pomegranate orchard in Tulare Co, CA and a citrus orchard at AgOps at UC Riverside. Fungal pathogens were isolated using larvae of the greater wax moth, *Galleria mellonella* L. and by soil plating on selective media. Waxworms were incubated in Petri plates with moist soil samples and fungal pathogens were isolated from cadavers. Alternatively, aliquots of soil suspensions were plated on media selective for *B. bassiana* and *Metarhizium anisopliae* (Metschnikoff) Sorokin. So far, 140 *B. bassiana* isolates and 4 *M. anisopliae* isolates have been isolated (Table 1). Additionally, *B. bassiana* was also isolated from the California harvester ant, *Pogonomyrmex californicus* Buckley, collected in Shafter, CA and the three-cornered alfalfa hopper, *Spissistilus festinus* (Say), collected in Parlier, California. Fungal isolates were cultured on selective and non-selective media to multiply the inoculum.

**Table 1.** Fungal pathogens isolated from citrus and pomegranate orchards and infected insects

Source	Method	<i>B. bassiana</i>	<i>M. anisopliae</i>
Organic citrus in Tulare Co	Waxworm bait	37	-
Pomegranate in Tulare Co	Waxworm bait	3	4
Riverside citrus	Waxworm bait	78	-
Riverside citrus	Selective media	22	-
California harvester ant	Selective medium	1	N/A
Three-cornered alfalfa hopper	Selective medium	1	N/A

### Pathogenicity of Entomopathogenic Fungi to GWSS

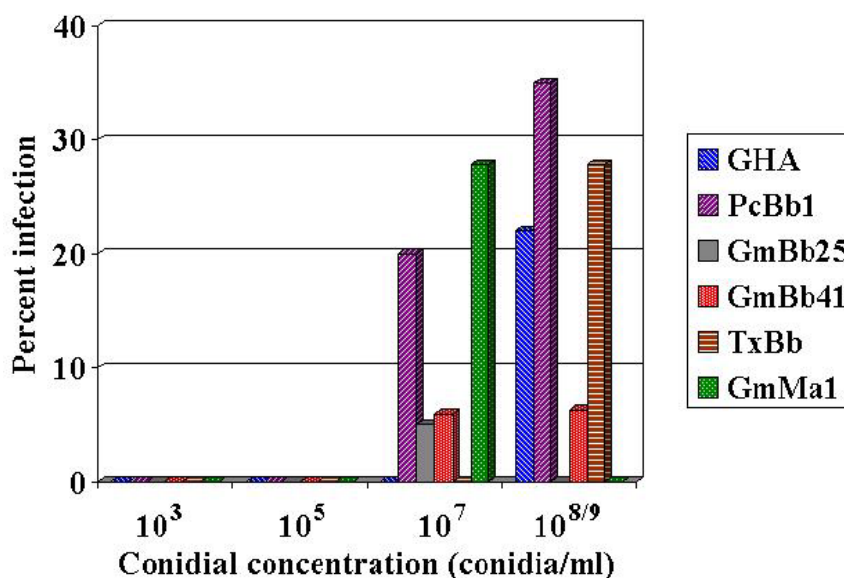
Laboratory-reared or field-collected GWSS adults supplied by CDFA, Arvin were used for the bioassays. GWSS were either placed at -5° C for 5 min or exposed to CO<sub>2</sub> for 15 sec to immobilize them and were inoculated by rolling them in a 10 µL drop of conidial suspension. Controls were treated with 0.01% of SilWet, an adjuvant used for preparing conidial suspensions. GWSS were individually incubated in a Petri plate with an excised citrus leaf and a moist filter paper. Petri plates were placed in a plastic box with moist paper towels and incubated at 27° C and 16:8 L:D photophase. GWSS were observed daily for mortality. Dead GWSS were surface sterilized in 3% sodium hypochlorite solution followed by rinsing in deionized water and incubated in sealed Petri plates on water agar or moist filter paper at 27° C in the dark.

#### Bioassay 1

The isolate of *B. bassiana* from *P. californicus* (PcBb1) was tested against laboratory-reared GWSS at four concentrations 10<sup>1</sup>, 10<sup>3</sup>, 10<sup>5</sup>, and 10<sup>7</sup> conidia/ml in comparison with controls. Each treatment and control had 10 adult GWSS. Infections were observed only at higher concentrations with 50% infection in GWSS treated with 10<sup>7</sup> conidia/ml and 10% in those treated with 10<sup>5</sup> conidia/ml.

#### Bioassay 2

Five *B. bassiana* isolates and a *M. anisopliae* isolate were tested against field-collected GWSS at four concentrations of 10<sup>3</sup>, 10<sup>5</sup>, 10<sup>7</sup>, and 10<sup>9</sup> (or 10<sup>8</sup> in case of *M. anisopliae*) conidia/ml along with untreated and SilWet (0.01%) treated controls. Isolates of *B. bassiana* included one from *P. californicus* (PcBb1), two from soil samples from citrus orchards in Tulare (GmBb25) and Riverside (GmBb41) counties, CA, one from *H. coagulata* in Weslaco, TX (TxBb) and a commercial isolate (designated GHA). The isolate of *M. anisopliae* (GmMa1) was from a soil sample from the pomegranate orchard in Tulare Co, CA. Each treatment and controls had 20 GWSS. Although all tested isolates were infective (Figures 1 and 2), all GWSS in this bioassay, including controls, suffered from a high mortality.



**Figure 1.** Pathogenicity of *B. bassiana* and *M. anisopliae* to GWSS



**Figure 2.** GWSS killed by *B. bassiana* and *M. anisopliae*.

### Bioassay 3

This assay was conducted using only  $10^9$  conidia/mL concentration and 10 laboratory-reared GWSS per isolate. All the isolates from the previous bioassay were used in this assay except for PcBb1, which was replaced by the *B. bassiana* isolate from *S. festinus* (SfBb1). This assay had also suffered from very high mortality and all the insects died within 5 days after the treatment. Fungal infection was seen in only one GWSS cadaver treated with SfBb1.

### CONCLUSIONS

The fact that GWSS is susceptible to entomopathogenic fungi such as *B. bassiana* is promising. Although infections occurred only at relatively high concentrations, there is enough variability in *B. bassiana* as a species to suggest other isolates may be more virulent. Efforts will continue to obtain isolates from collaborators and from likely GWSS host habitat in California for further laboratory evaluation and eventual field application.

### REFERENCES

- Galaini-Wraight, S., S.P. Wraight, R.I. Carruthers, B.P. Magalhaes, and D.W. Roberts. 1991. Description of a *Zoophthora radicans* (Zygomycetes, Entomophthoraceae) epizootic in a population of *Empoasca kraemeri* (Homoptera, Cicadellidae) on beans in central Brazil. *J. Invertebr. Pathol.* 58: 311-326.
- Grafton-Cardwell, E.E. and C. Kallsen. 2001. Efficacy of insecticides used for glassy-winged sharpshooter control in citrus. *Pierce's Disease Research Symposium*, Dec 5-7, 2001. pp. 32-34.
- Hywel-Jones, N.L., H.C. Evans, and Y. Jun. 1997. A re-evaluation of the leafhopper pathogen *Torribiella hemipterigena*, its anamorph *Verticillium hemipterigenum* and *V. pseudohemipterigenum* sp. nov. *Mycol. Res.* 101: 1242-1246.
- Magalhaes, B.P., R.A. Humber, E.J. Shields, and D.W. Roberts. 1991. Effects of environment and nutrition on conidium germination and appressorium formation by *Zoophthora radicans* (Zygomycetes, Entomophthorales): a pathogen of the potato leafhopper (Homoptera, Cicadellidae). *Environ. Entomol.* 20: 1460-1468.
- Matsui, T., H. Sato, and M. Shimazu. 1998. Isolation of an entomogenous fungus, *Erynia delphacis* (Entomophthorales: Entomophthoraceae), from migratory planthoppers collected over the Pacific Ocean. *Appl. Entomol. Zool.* 33: 545-549.
- McGuire, M.R., M.J. Morris, E.J. Amburst, and J.V. Maddox. 1987. An epizootic caused by *Erynia radicans* (Zygomycetes: Entomophthoraceae) in an Illinois *Empoasca fabae* (Homoptera: Cicadellidae) population. *J. Invertebr. Pathol.* 50: 78-80.
- Mizell, R.F. and D.G. Boucias. 2002. Mycopathogens and their exotoxins infecting glassy-winged sharpshooter: survey, evaluation, and storage. *Pierce's Disease Research Symposium*, Dec 15-18, 2002. pp. 161-162.
- Wells, J.M., B.C. Raju, H.Y. Hung, W.G. Weisburg, L. Mandelco-Paul and D.J. Brenner. 1987. *Xylella fastidiosa* gen. nov., sp. nov.: gram-negative, xylem-limited, fastidious plant bacteria related to *Xanthomonas* spp. *Int. J. Syst. Bacteriol.* 37: 136-143.

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# IDENTIFICATION OF MECHANISMS MEDIATING COLD THERAPY OF *XYLELLA FASTIDIOSA*-INFECTED GRAPEVINES

## Project Leader:

Bruce Kirkpatrick  
Dept. of Plant Pathology  
University of California-Davis  
Davis, 95616

## Cooperator

Melody Meyer  
Dept. of Plant Pathology  
University of California-Davis  
Davis, 95616

**Reporting Period:** The results reported here are from work conducted from July 2004 to November 2004.

## ABSTRACT

Preliminary xylem sap composition studies were conducted in February 2004 using Cabernet sauvignon and Pinot noir grapevines growing in Placerville (cold winter temperature) and UC Davis (warmer temperatures). The pH of xylem sap from both varieties was almost a full unit lower in vines grown in cold temperatures versus warm. A similar trend also occurred with sap osmolarity, however the differences were not as great. Because these vines were grown under different management practices and on different rootstocks these results must be considered preliminary. In 2004 we established four field sites in Shasta, Placer, Mendocino and Yolo counties to repeat these measurements on clonal vines that were grown in 5-gallon pots at University of California, Davis. One-half of the vines were inoculated with *Xf* while the other half is uninoculated controls. Sap will be collected from the vines during the late winter and pH, osmolarity, carbohydrates, organic acids and abscisic acid (ABA) will be measured and compared. The vines will be returned to University of California, Davis at bud break and observed for the development of PD symptoms and tested by PCR to determine if any of the vines were "cold cured" of their infection. Similar experiments using potted vines that will be exposed to defined cold temperature regimes in cold storage facilities located at University of California, Davis will be conducted in 2005. Proteins present in the collected xylem sap will be analyzed by PAGE and the identity of major or unique xylem sap proteins will be determined by sequencing them. *Xf* viability studies using buffers of various pHs, xylem sap from warm- and cold-treated vines will also be studied. The goal of this research is to understand the physiological/biochemical basis of cold therapy that was first documented by A.H. Purcell.

## INTRODUCTION

The geographical distribution of Pierce's disease (PD) in North America is strongly associated with the severity of winter temperatures, i.e. PD does not occur in New York, the Pacific Northwest nor at high altitudes in S. Carolina, Texas and even California (Hopkins and Purcell, 2002). Sandy Purcell demonstrated that relatively brief exposures to sub-freezing temperatures can eliminate *Xylella fastidiosa* in some percentage of cold treated *V. vinifera* grapevines, however some of the coldest temperatures he used killed the vines (Purcell 1977, 1980). He also found that a higher percentage of vines that were moderately susceptible to PD such as Cabernet sauvignon, were cured by cold therapy treatments compared to susceptible varieties such as Pinot noir. Purcell's group also showed that whole, potted vines exposed to low temperatures had a higher rate of recovery than PD-affected, detached bud sticks exposed to the same cold temperatures (Feil, 2002). Clearly, some factor(s) that were expressed in the intact plant, but not in detached bud sticks, helped eliminate *Xf* from the plants. Our objective is to elucidate the physiological/biochemical basis that mediates cold therapy and to identify the physiological/biochemical factor(s) that occur or are expressed in cold treated vines that eliminate *Xf*. If such factor(s) are found, it may be possible to induce their expression under non-freezing temperatures and potentially provide a novel approach for managing PD.

## OBJECTIVES

1. Develop an experimental, growth chamber temperature regime that can consistently cure Pierce's disease affected grapevines without causing unacceptable plant mortality.
2. Analyze chemical changes such as pH, osmolarity, total organic acids, proteins and other constituents that occur in the xylem sap of cold-treated versus non-treated susceptible and less susceptible *Vitis vinifera* varieties.
3. Assess the viability of cultured *X. fastidiosa* cells growing in media with varying pH and osmolarity and cells exposed to xylem sap extracted from cold- and non-treated grapevines.
4. Determine the effect of treating PD-affected grapevines with cold plant growth regulators, such as abscisic acid (ABA), as a possible therapy for PD.

## RESULTS AND CONCLUSIONS

### Objective 1

The same varieties used by Purcell (1977, 1980) and Feil (2002) in previous cold therapy studies, Pinot noir (PD-susceptible) and Cabernet sauvignon (moderately resistant to PD) grapevines grafted on 101-14 rootstock were inoculated with *Xf* in the spring of 2004 using a pinprick inoculation procedure (Hill and Purcell, 1995; Purcell and Saunders, 1999). The vines were grown in five gallon pots in a greenhouse using a nutrient-supplemented irrigation regime. Treatment vines were inoculated with the Stagg's Leap strain of *Xylella fastidiosa*, whereas control vines were inoculated with water. During late summer and fall, the plants were moved into a screen house in order to acclimatize them to decreasing temperatures. While in the screen

house, plants were watered by drip irrigation and supplemental fertilizer application until the first week of October 2004. Twelve weeks after inoculation, the plants were rated for symptom development.

During October/November, 2004, 11 inoculated and 11 controls of each variety (44 plants total) were transported to 3 sites that were selected because of their relatively cold winter temperatures, as well as University of California, Davis which was the control. Plot sites include: Fall River (Shasta County), University of California Hopland Research Station (Mendocino County), and University of California, Blodgett Forest Research Station (Placer County). Potted grapevines were planted in the ground to the top of the pot in order to maintain uniform soil type, prevent roots in the pots from exposure to abnormally cold temperatures, and to prevent the plants from falling over. Plants were irrigated as needed until rain provided adequate moisture for the vines. Vines will be allowed to undergo natural dormancy during the fall and experience ambient temperatures during the winter. Temperature, ETo, and other weather data for each plot are being monitored using CIMIS weather data (<http://www.cimis.water.ca.gov/cimis/data.jsp>). This data, and previous temperature profiles at these sites, will be used to determine a growth chamber temperature regime that can consistently cure PD affected grapevines without causing unacceptable plant mortality. Additional grapevines, using the same varieties and inoculated as described above, but grown in 6 inches standard pots will be exposed to different temperature regimes in cold rooms located at the Department of Pomology, University of California, Davis during the winter/spring of 2005.

### **Objective 2**

Preliminary work from Pinot noir and Cabernet sauvignon field materials collected from Placer and Yolo counties showed some differences in xylem sap pH and osmolarity. These results were obtained from Pinot noir and Cabernet sauvignon vines growing in one Placerville vineyard and at a vineyard at University of California Davis. Both varieties were grown in the same manner at each site, however management practices at the two sites were not identical. It is also important to note that the University of California Davis vines were grown on 5C rootstocks while the Placerville vines were not grown on rootstocks and that these vines were not the same clones. Dormant cuttings were collected in late February and xylem sap was extracted using a custom-made pressure bomb. Differences were noted in xylem sap pH, abscisic acid concentration, and osmolarity. These same parameters will be further examined in 2005 in the field sites and growth chamber experiments. Although only preliminary findings, we found that the pH of xylem sap collected in late February was lower, 5.37 for Pinot and 5.23 for Cabernet vines at the Placerville site (colder winter temperatures) than vines growing at University of California Davis, 6.35 and 6.06, respectively. Small differences in osmolarity were also noted in xylem sap from Placerville, 55.2 and 55.5, versus the osmolarity of xylem sap from Davis vines, 58.3 and 60.8 respectively. The significance and reproducibility of these differences needs to be confirmed this winter using the more controlled experimental units.

During the 2005 winter months, field grown and growth chamber plants will be sampled for potential changes in pH, osmolarity, total organic acids, proteins and other constituents that occur in xylem sap. Our hypothesis is that changes in xylem sap components in vines that undergo cold treatment may have significant effects on *Xf* viability. Previous research on several plant species has shown that a number of plant genes are expressed in response to freezing temperatures (reviewed by Thomashow, 1998). In some plants, these freeze-induced proteins are structurally related to proteins that plants produce in response to pathogens, i.e. pathogenesis-related proteins (Hon, et al. 1995; Kuwabara, et al, 2002). Thus it maybe possible that cold-stressed grapevines could produce proteins that are deleterious to *Xf*. To investigate this possibility, xylem sap will be expressed from cold-stressed and control vines using the pressure bomb, concentrated by freeze drying, and protein profiles determined by 1 and 2 dimensional polyacrylamide gel electrophoresis (PAGE). If unique proteins are found in the cold stressed plants these proteins will be cut from the gel, end terminally sequenced by the University of California Molecular Structure Facility and their sequences compared to others in the database. The potential effect of these proteins on *Xf* viability will be assessed as described in Objective 3.

### **Objective 3**

We have been assessing the effect of many of the physical, physiological and biochemical parameters we determined in Objective 1 and 2 on *Xf* viability. We have been assessing the effect of pH and osmolarity on the viability of *Xf* cells *in vitro* using various buffers and media such as PD3 and new chemically defined media (Leite, et al., 2004). The liquid solutions used for these viability experiments included: water, extracted xylem sap, PD3, the Leite medium, HEPES, sodium and potassium phosphate buffers. In order to further examine these parameters, cultures of *X. fastidiosa* Stagg's Leap strain were grown at 28°C on PD3 for 11 days. Cells were scraped from the culture plates and suspended at concentrations of  $1.5 \times 10^7$  bacteria per mL of liquid medium. One mL of the suspension was then placed into each 1.5 mL microcentrifuge tubes and placed at various temperatures. Samples were diluted and plated out onto PD3 and allowed to grow for seven days. After seven days, colonies were counted to determine the potential effect each treatment had on the viability of *Xf* cells. Results of these experiments indicate that *Xf* can survive at -5°C for 8 weeks. At lower temperatures, our results were similar to those found by Feil (2002). *Xf* survived the best in HEPES and sodium phosphate buffers and the worse survival occurred in waters and xylem sap at -5°C. At -10 and -20°C *Xf* rapidly died in all liquid media tested.

We also adjusted the pH of potassium phosphate buffer to the values determined for cold-stressed and control xylem saps collected from Placerville and University of California, Davis vines described previously. Cultures of *X. fastidiosa* Stagg's Leap strain were again grown at 28°C on PD3 for 11 days. Cells were harvested from culture plates and suspended at

concentrations of  $1.5 \times 10^7$  bacteria per mL of potassium phosphate buffer. One mL of suspension was then placed into each 1.5 mL microcentrifuge tubes and placed at  $-5^{\circ}\text{C}$ . Samples were diluted and plated out onto PD3 and allowed to grow for seven days. After seven days colonies were counted to determine the effect of pH on the viability of the *Xf* cells. *Xf* survived the best in potassium phosphate at pH 6.6 and 6.8 and the poorest survival occurred at pH 5.0. There was significant variation between reps of these experiments so they are now being repeated; however it is interesting that these initial trends are consistent with the pH values of xylem saps extracted from Placerville, where PD is not known to occur, and saps from vines growing at Davis where *Xf* can overwinter in grapevines.

#### **Objective 4**

Previous research has shown that herbaceous and woody plants exposed to sub-lethal cold conditions have significantly elevated levels of plant hormones, such as abscisic acid (ABA), which induces the synthesis of a number of cold shock proteins (Bravo, et al., 1998; Thomashow, 1998). Preliminary studies, involving samples of Pinot noir and Cabernet sauvignon field materials collected from Placer and Yolo counties in February, 2004, showed abscisic acid concentrations were lower in the Placerville, cold-exposed vines, than vines from Davis. ABA concentrations were lower in Pinot than Cabernet for both Placerville and Davis vines. Again, it will be important to verify these initial findings using vines grown under more controlled environments in growth chambers during 2005.

We will determine the concentration of ABA in cold-stressed and control vines growing both in the growth chamber using the temperature regimes determined in Objective 1 and in the field-grown plants in the four sites described in Objective 1. We will also determine the pH, osmolarity and protein profiles of xylem sap from ABA-treated vs. non-treated vines and assess the potential of this sap for anti-*Xf* activity.

During the spring, summer and fall, Cabernet and Pinot vines will be sprayed with 100uM solutions of ABA, a concentration that elicited cold-shock proteins at  $23^{\circ}\text{C}$  in winter wheat (Kuwabara, et. al 2002). Additional concentrations up to 500uM may also be evaluated if no response is noted at 100uM. The pH and osmolarity of xylem sap from the treated vines will be determined as described above. The concentration of ABA in the sap will be determined using a commercially available immunoassay that has a sensitivity of 0.02-0.5 picomole/0.1 mL (Plant Growth Regulator Immunoassay Detection Kits, Sigma Chemical Co.). Preliminary work has shown that ABA concentrations in grapevine xylem sap are detectable using this kit. Xylem sap proteins will be collected, concentrated and analyzed by 1 and 2 dimensional PAGE as previously described. Unique proteins expressed in ABA-treated vines will be removed from the gels and end terminally sequenced and analyzed as previously described.

#### **REFERENCES**

- Bravo, L.A., Zuniga, G.E., Alberdi, M., and Carcuera, L.S. 1998. The role of ABA in freezing tolerance and cold acclimation in barley. *Physiol. Plant.* 103: 17-23.
- Feil, H., 2002. Effect of sub-freezing temperature on the survival of *Xylella fastidiosa* in vitro and in plants. Ph.D. dissertation, University of California, Berkeley.
- Hill, B.L. and Purcell, A.H. 1995. Multiplication and movement of *Xylella fastidiosa* within grapevine and four other plants. *Phytopathol.* 85: 1368-1372.
- Kuwabara, C., Takezawa, D., Shimada, T., Hamada, T., Fujikawa, S., and Arakawa, K. 2002. Absciscic acid- and cold-induced thaumatin-like protein in winter wheat has an antifungal activity against snow mold, *Microdochium nivale*. *Physiol. Plant.* 115: 101-110.
- Purcell, A.H. 1977. Cold therapy of Pierce's disease grapevines. *Plant Dis. Repr.* 61: 514-518.
- Purcell, A.H. 1980. Environmental therapy for Pierce's disease of grapevines. *Plant Dis.* 64: 388-390.
- Purcell, A.H. and Saunders, S.R. 1999. Fate of Pierce's disease strains of *Xylella fastidiosa* in common riparian plants in California. *Plant Dis.* 83: 825-830.
- Thomashow, M.F. 1998. Role of cold responsive genes in plant freezing tolerance. *Plant Physiol.* 118:1-7.

#### **FUNDING AGENCIES**

Funding for this project was provided by the CDFA Pierce's Disease and Glass-winged Sharpshooter Board.



**SYMBIOTIC CONTROL OF PIERCE'S DISEASE: CONSTRUCTION OF TRANSGENIC STRAINS  
OF *ALCALIGENES XYLOSOXIDANS DENITRIFICANS* EXPRESSING SURFACE ANTI-*XYLELLA* FACTORS  
AS MICROBIAL PESTICIDES FOR PIERCE'S DISEASE CONTROL**

**Project Leader:**

David Lampe  
Dept. of Biological Sciences  
Duquesne University  
Pittsburgh, PA 19219

**Project Director:**

Thomas A. Miller  
Dept. of Entomology  
University of California  
Riverside, CA 92521

**Cooperators:**

Carol Lauzon  
Dept. of Biological Sciences  
California State University  
Hayward, CA 94542

Don Cooksey  
Department of Plant Pathology  
University of California  
Riverside, CA 92521

Blake Bextine  
Dept. of Entomology  
University of California  
Riverside, CA 92521

Steven Lindow  
Dept. of Plant and Microbial Biology  
University of California  
Berkeley, CA 94720

**Consultant:**

Frank Richards  
Yale University  
New Haven, CT 06520

**Reporting period:** The results reported here are from research conducted from April 2003 to October 2004.

**INTRODUCTION**

The glassy-winged sharpshooter (GWSS) is the principle vector of the xylem-limited bacterium *Xylella fastidiosa* (*Xf*), which causes Pierce's disease (PD) in grapes. Limiting the spread of this pathogen by rendering GWSS incapable of pathogen transmission (paratransgenesis) is a promising method of pathogen control. Paratransgenesis seeks to modify the phenotype of an organism indirectly by modifying its symbiotic bacteria to confer vector-incompetence

Paratransgenic approaches to disrupt pathogen infection of humans are being developed by several groups. These include interference with the ability of triatomid bugs to transmit pathogens causing Chagas' disease<sup>1</sup>, interference with HIV attachment to its target cells in the reproductive tracts of humans<sup>2</sup>, and the elimination of persistent *Candida* infections from biofilms in chronically infected patients<sup>3</sup>. Paratransgenesis has also been applied to deliver cytokines mammalian guts to relieve colitis<sup>4,5</sup>. Thus, the method has wide applicability.

*Alcaligenes xylosoxidans denitrificans* (*Axd*) is Gram negative, beta proteobacterial species that can colonize the GWSS foregut and cibarium, as well as various plant tissues, including xylem. It is non-pathogenic in insects, plants and healthy humans. Given these characteristics, *Axd* has become the focus of our paratransgenesis efforts to control PD in grapes. Over the past two years we developed the technology to stably modify *Axd* by inserting genes into its chromosome and also isolated as single chain antibody that recognized an epitope on the surface of the Pierce's Disease strain of *Xf*.<sup>6</sup>

We report here the construction of strains of *Axd* that express an anti-*Xylella* single chain antibody (scFv) on the outer surface of *Axd* as fusions to three different heterologous outer membrane proteins. In each case, strains of varying fitness were recovered as measured by growth rate as compared to wild-type strains.

**OBJECTIVES**

1. Construct anti-*Xylella* scFv-membrane protein fusions;
2. Construct strains of *Axd* that express the scFv-membrane protein fusions in the outer membrane;
3. Construct transgenic *Axd* strains of varying fitness.

**RESULTS**

**A. Membrane Protein-scFv Gene Fusions**

We fused an anti-*Xylella* scFv gene to three different outer membrane protein genes in order to display the scFv on the outer membrane of *Axd*. These were a lipoprotein-outer membrane protein A (*lpp-OmpA*) fusion from *E. coli*<sup>7</sup>; the ice nucleation protein Z (*inaZ*) from *Pseudomonas syringae* (a gift of Steven Lindow); and an internally-deleted form of *inaZ* that eliminates the internal ice nucleation repeat sequence but retains the N and C terminus of the protein necessary to export and

anchor it in the outer membrane (short-*inaZ*)<sup>8</sup>. Each of these was placed on a *HimarI mariner* transposon and random chromosomal insertions were obtained for each generating multiple strains<sup>9</sup> (see Table 1).

### B. Expression of scFv Fusions on the Surface of *Axd*.

We determined the degree of surface expression of the scFv fusions on *Axd* by two methods. The first was a “spun cell ELISA”<sup>7</sup>. This method uses a suspension of cells that express a target epitope as the substrate for an ELISA. Detection of the scFv was accomplished before and after induction of the *lac* promoter by either reaction with Protein L-conjugated HRP (which detects scFv light chains) or with a HRP conjugated antibody that reacts with the haemagglutinin epitope tag on the scFv. Results of spun cell ELISAs on different strains are shown in Table 1. Strains varied considerably in their scFv surface expression levels, presumably due to the site of insertion. Most strains of short-*inaZ* fusions, for example were poor expressers when induced and strain AL8.2 only showed appreciable levels of surface expression when uninduced.

**Table 1.** Characteristics of transgenic *A. xylosoxidans* strains expressing an anti-*Xylella* single chain antibody as an outer membrane protein fusion.

Strain	scFv fusion	Surface expression <sup>1</sup>	Relative Fitness <sup>2</sup>	Insert location <sup>3</sup>
AL7.2	<i>P. syringae inaZ</i>	++	G	- major facilitator superfamily transporter
AL7.5	“”	+++	G	- inorganic pyrophosphatase
AL7.7	“”	++	S	-fructose transport system repressor
AL7.10	“”	+	G	ND
AL8.2	<i>P. syringae</i> short <i>inaZ</i>	+++ (uninduced only)	G/P	-probable transporter
AL8.3	“”	BK	P	ND
AL9.1	<i>E. coli lpp-ompA</i>	+	S	ND
AL9.4	“”	+++	S/G	ND
AL9.5	“”	+	S	ND

<sup>1</sup> These values are relative to background as measured in a spun cell ELISA: BK = background levels; + noticeable expression, ++ strong expression; +++ very strong expression.

<sup>2</sup> Fitness values are measured as growth rates in liquid culture relative to that of wild-type *A. xylosoxidans*. S (= strong, essentially wild type); G (= good, but slower than wild type); P (= poor)

<sup>3</sup> Most likely identity of genes where transgenes were inserted. These were obtained using tblastx with flanking insertion sequences against the microbial nucleotide database from Genbank. ND= not determined.

The second method used to determine whether expression was occurring in the outer membrane of *Alcaligenes* was a test for ice nucleation. Wild-type *Axd* cannot nucleate ice (unpublished observations). We tested whether or not AL7 and AL8 strains could nucleate ice. All of the AL7 strains could nucleate ice while neither of the AL8 strains did so. This is consistent with surface expression of the full-length *P. syringae* ice nucleation protein on the surface of the AL7 strains. AL8 strains express a form of *inaZ* that has the internal repeat region removed. This is the region that is responsible for ice nucleation in these proteins.

### C. Fitness of transgenic *Axd* strains

Our strains are built via transposon insertion and so should vary in fitness depending on the site of insertion in the chromosome. We measured the fitness of each strain compared to wild type by measuring their growth rates in log phase in liquid culture. These relative fitness values are shown in table 1 along with the most likely site of insertion of the transposon used to make the strain. We determined the site of insertion by sequencing outward from the transposable element inverted terminal repeats into the flanking genomic DNA and then using tblastx against the microbial genomic database in Genbank. There are no *Axd* sequences in Genbank, so the matches we obtained were typically to species in the genus *Pseudomonas*, another basal beta proteobacterial group.

Strains were highly variable in their fitnesses. Some strain fitnesses were indistinguishable from wild type (e.g., AL7.7 and AL9.5), while others were obviously affected in their growth rates (e.g., AL8.3). There was no obvious correlation between fitness and ability to surface express the scFv fusions. Indeed, one of our best expressing strains was only a modest grower (AL7.5) while other strains grew well and expressed the transgene poorly (e.g., AL9.5). The ability to isolate strains that



vary in fitness is an important aspect of paratransgenesis since we are interested in providing *Axd* reagents that vary in their level of persistence.

#### **D. Determining the target of the anti-*Xylella* scFv**

We attempted to determine the target of the anti-*Xylella* scFv we isolated previously. We used a combination of 1-D and 2-D SDS-PAGE gels and western blotting to determine a size range for the target protein.

### **CONCLUSIONS**

We have created multiple transgenic strains of the plant and insect symbiotic bacterium, *Alcaligenes xylosoxidans* (*denitrificans*) that carry a surface expressed anti-*Xylella* antibody. These strains carry chromosomal insertions of the genes for the scFv and we were able to recover strains that varied in fitness and in their expression level for the scFv on their outer membranes. These initial strains are currently being tested for their ability to interfere with the transmission of *X. fastidiosa* by sharpshooters.

The future goals of this project are to isolate new anti-*Xylella* factors that can be expressed on the surface of *Axd*, to incorporate genetic systems aimed at preventing horizontal gene transfer of the transgenes, and to improve expression levels of the transgenes on the surface of the cell. All of these features are aimed at developing strains of *Axd* that can interrupt the spread of *Xylella* from the glassy-winged sharpshooter to uninfected grapevines.

### **REFERENCES.**

- Beard, C. B., Dotson, E. M., Pennington, P. M., Eichler, S., Cordon-Rosales, C. & Durvasula, R. V. (2001). Bacterial symbiosis and paratransgenic control of vector-borne Chagas disease. *Int J Parasitol* 31, 621-7.
- Chang, T. L., Chang, C. H., Simpson, D. A., Xu, Q., Martin, P. K., Lagenaur, L. A., *et al.* (2003). Inhibition of HIV infectivity by a natural human isolate of *Lactobacillus jensenii* engineered to express functional two-domain CD4. *Proc Natl Acad Sci U S A* 100, 11672-7.
- Beninati, C., Oggioni, M. R., Boccanera, M., Spinosa, M. R., Maggi, T., Conti, S., *et al.* (2000). Therapy of mucosal candidiasis by expression of an anti-idiotypic in human commensal bacteria. *Nat Biotechnol* 18, 1060-4.
- Steidler, L., Hans, W., Schotte, L., Neirynck, S., Obermeier, F., Falk, W., *et al.* (2000). Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. *Science* 289, 1352-5.
- Steidler, L. (2001). *Lactococcus lactis*, A Tool for the Delivery of Therapeutic Proteins Treatment of IBD. *ScientificWorldJournal* 1, 216-7.
- Bextine, B., Lauzon, C., Potter, S., Lampe, D. & Miller, T. A. (2004). Delivery of a genetically marked *Alcaligenes* sp. to the glassy-winged sharpshooter for use in a paratransgenic control strategy. *Curr Microbiol* 48, 327-31.
- Benhar, I., Azriel, R., Nahary, L., Shaky, S., Berdichevsky, Y., Tamarkin, A., *et al.* (2000). Highly efficient selection of phage antibodies mediated by display of antigen as Lpp-OmpA' fusions on live bacteria. *J Mol Biol* 301, 893-904.
- Lee, S. Y., Choi, J. H. & Xu, Z. (2003). Microbial cell-surface display. *Trends Biotechnol* 21, 45-52.
- Rubin, E. J., Akerley, B. J., Novik, V. N., Lampe, D. J., Husson, R. N. & Mekalanos, J. J. (1999). *In vivo* transposition of *mariner*-based elements in enteric bacteria and mycobacteria. *Proc Natl Acad Sci U S A* 96, 1645-1650.

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**SYMBIOTIC CONTROL OF PIERCE'S DISEASE: THE BIOLOGY OF THE SHARPSHOOTER SYMBIONT,  
*ALCALIGENES XYLOSOXIDANS* SUBSP. *DENITRIFICANS***

**Project Leader:**

Carol Lauzon  
Dept. of Biological Science  
California State University  
Hayward, CA 94542

**Project Director:**

Thomas Miller  
Dept. of Entomology  
University of California  
Riverside, CA 92521

**Cooperators:**

David Lampe  
Biology Dept.  
Duquesne University  
Pittsburgh, PA 19219

Don Cooksey  
Dept. of Plant Pathology  
University of California  
Riverside, CA 92521

Steven Lindow  
Dept. of Plant and Microbial Biology  
University of California  
Berkeley, CA 94720

Blake Bextine  
Dept. of Entomology  
University of California  
Riverside, CA 92521

**Graduate Students:**

Lavanya Telukuntla  
Dept. of Biological Science  
California State University  
Hayward, CA 94542

Ranjana Ambannavar  
Dept. of Biological Science  
California State University  
Hayward, CA 94542

**Reporting period:** The results reported here are from work conducted from April 2003 to October 2004.

**ABSTRACT**

*Alcaligenes xylosoxidans denitrificans* (Axd) is closely associated with *Homalodisca coagulata*, the glassy-winged sharpshooter (GWSS), and xylem fluid of host plants. The bacterium has long been characterized as a nitrogen and hydrogen recycler in nature, and was recently recognized as an important decomposer of cyanogenic glycosides in plant material (Ingvorsen et al. 1991). Few studies exist that describe the fitness of Axd when it is introduced to competitive environments, such as established soil or plant microbial communities. Such studies lend important information for assessment of the potential use of Axd for symbiotic control of *Xylella fastidiosa*, the causal agent of Pierce's disease. We have found that Axd and Axd containing DsRed fluorescent protein (Raxd) do not establish when introduced into soil, but can be recovered from soil that was sterilized before inoculation with Axd or Raxd. Axd and Raxd can also be recovered from established phylloplane communities of basil, strawberry, and sage, although recovery is scant to low. Current studies underway include the recovery of Axd and Raxd from lake water microbial communities. Co-culture experiments showed that Axd and Raxd growth is negatively affected by the presence of *Escherichia coli* and the pathogen *Pseudomonas aeruginosa*. Raxd was modified to express an S1 scFv (single chain antibody variable region fragments) antibody (Axd 7.7) that binds specifically to a strain of *X. fastidiosa* that infects grape. Axd 7.7 growth in culture was compared to that of the wild type Axd and to Raxd. All strains exhibited similar growth patterns in tryptic soy broth (TSB). All strains demonstrated longer lag phases in Luria Bertani medium (LB) than for TSB. Cell numbers remained fairly constant for each strain at each growth phase. Growth studies are underway that monitor the growth of Axd, Raxd, and Axd 7.7 in dilute, R2A medium. Current studies also include using enzyme linked immunosorbent assays to monitor the expression of S1 scFv from Axd 7.7 under environmental challenges, such as poor nutrient availability and energetic demands.

**INTRODUCTION**

*Alcaligenes xylosoxidans* subsp. *denitrificans* (Axd) is currently being tested for use in symbiotic control of Pierce's disease. While the bacterium naturally resides in terrestrial and aquatic environments, little is known about the fitness of Axd when it is artificially introduced to either allocthonous or autocthonous environments with established microbial communities. Therefore, some indication of the fitness of Axd in competitive biotic scenarios must be acquired to begin to assess the potential of Axd to control *Xylella fastidiosa* (Xf) under natural conditions. This point also holds true for any strain of Axd that is modified to express anti-Xf products. In most cases, a genetically modified bacterium (GMB) is less fit than the wild type counterpart (Velicer, 1999). In an ideal case, a GMB should remain in an ecosystem for a limited but effective period of time and cause minimal or no disruption to a host or ecosystem. Here we report on the recovery of Axd and Raxd when introduced onto plant surfaces and in soil using semi-natural experimental conditions. In addition, we provide information regarding the growth of Axd and Raxd when grown under strict laboratory conditions in the presence of human and plant-associated bacteria. We also provide a comparison of the growth of Axd, Raxd, and Axd genetically modified to express a synthetic antibody construct on its cell surface (Axd 7.7) under different growth conditions.

## OBJECTIVES

1. Study the behavior of strains of *A. xylosoxidans* subsp. *denitrificans* (Axd and Raxd) when grown under various biotic influences and,
2. Investigate and compare the growth of *A. xylosoxidans* subsp. *denitrificans* (wild type) and Raxd to that of Axd modified to express a short chain antibody against *X. fastidiosa* (Axd 7.7) that infects grape under different physiological conditions, such as in response to nutrient availability and energetic demands.

## RESULTS

We have found that Axd and Raxd do not establish when introduced into soil, but can be recovered from soil that was sterilized before inoculation with Axd and Raxd. Axd and Raxd, when applied to leaf surfaces, can be recovered from established phylloplane communities of basil, strawberry, and sage, although recovery is scant to low. Co-culture experiments showed that Axd and Raxd growth are negatively affected by the presence of *E. coli* and *P. aeruginosa*. The growth of Axd modified to express an S1 scFv (single chain antibody variable region fragments) antibody (Axd 7.7) that binds specifically to a strain of *X. fastidiosa* that infects grape was compared to that of the wild type Axd and Raxd. Axd, Raxd, and Axd 7.7 exhibited similar growth patterns in tryptic soy broth (TSB). Axd, Raxd, and Axd 7.7 also demonstrated longer lag phases in Luria Bertani medium (LB) than for TSB. Cell numbers remained fairly constant for each strain at each growth phase. Growth studies are underway that monitor the growth of Axd, Raxd, and Axd 7.7 in dilute, R2A medium. Current studies also underway include using enzyme linked immunosorbent assays to monitor the expression of S1 scFv from Axd 7.7 under environmental challenges, such as poor nutrient availability and energetic demands.

## CONCLUSIONS

From earlier work we have found that Raxd establishes within the mouthparts of *H. coagulata* (Bextine et al. 2004a) and within the xylem of several of this sharpshooter's host plants (Bextine et al. 2004b). The bacterium, however, does not establish within soil if soil communities are in place. If the soil is sterilized and biotic competition is eliminated, then Axd and Raxd grow relatively well. Conversely, Axd and Raxd can survive and be retrieved from the leaf surfaces of plants other than citrus, such as basil, sage, and strawberry plants for up to two weeks. These data suggest that Axd and Raxd are more suited to the plant environment than to a soil environment. We conclude that Axd and Raxd will remain in the plant environment long enough to exert an anti-*Xylella* effect with little to no disruption of any relevant ecosystem. Raxd did not grow well in the presence of *E. coli* and *P. aeruginosa* compared to Raxd grown in pure culture. Thus, compared to a ubiquitous bacterium and a pathogen, respectively, Raxd is not as fit under standard growth conditions.

Axd 7.7 growth compared to Axd and Raxd differed little under our experimental conditions. All data collectively suggest that Axd 7.7 shows potential for delivery of an anti-*Xylella* product with little impact on nontarget bacterial ecosystems. This statement is qualified by the fact that field tests must be implemented to assess the true behavior of strains of Axd in the environment. Laboratory studies are not suitable for a genuine assessment of risk assessment and environmental impact; nevertheless, they provide important insight.

## REFERENCES

- Bextine, B.R., C.R. Lauzon, D. Lampe, and T.A. Miller. 2004a. Delivery of a genetically marked *Alcaligenes* sp. to the glassy-winged sharpshooter for use in a paratransgenic control strategy. *Curr. Microbiol.* 48: 327-331.
- Bextine, B.R., C.R. Lauzon, D. Lampe and T.A. Miller. 2004b. Establishment of a genetically marked insect-derived symbiont in multiple host plants. *Curr. Microbiol. In Press.*
- Ingvorsen, K., B. Højer-Pedersen, and S. E. Godtfredsen. 1991. Novel cyanide-hydrolyzing enzyme from *Alcaligenes xylosoxidans* subsp. *denitrificans*. *Appl. Environ. Microbiol.* 57: 1783-1789.
- Velicer, G.J. 1999. Pleiotropic effects of adaptation to a single carbon source for growth on alternative substrates. *Appl. Environ. Microbiol.* 65(1): 264-269.

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# MANAGEMENT OF PIERCE'S DISEASE OF GRAPE BY INTERFERING WITH CELL-CELL COMMUNICATION IN *XYLELLA FASTIDIOSA*

## Project Leader:

Steven E. Lindow  
Dept. of Plant and Microbial Biology  
University of California  
Berkeley, CA 94720

## Cooperators:

Karyn Lynn Newman  
Dept. of Plant and Microbial Biology  
University of California  
Berkeley, CA 94720

Alexander Purcell  
Dept. of Environmental Science Policy & Management  
University of California  
Berkeley, CA 94720

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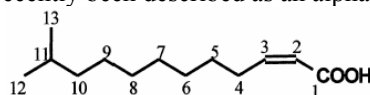
## ABSTRACT

*Xylella fastidiosa* (*Xf*) is an endophyte that is restricted to the xylem, a network of vessels for water transport, in which it forms an aggregated biofilm. It is transmitted from plant to plant by xylem sap-feeding insects, and forms a polar biofilm in these insects' foreguts. In other systems, biofilms are characterized by community behavior under the control of cell density-dependent gene expression, which requires cell-cell signaling. *Xf* has homologs of the cell-cell signaling genes found in the important plant pathogen *Xanthomonas campestris* pathovar *campestris* (*Xcc*) and produces a similar alpha,beta unsaturated fatty acid signal molecule called DSF that coordinates gene expression in a community (2, 7). We have investigated DSF-mediated cell-cell signaling in *Xf* with the aim of developing cell-cell signaling disruption as a means of controlling Pierce's disease. We have determined that the *rpjF* gene is necessary and sufficient for DSF signal synthesis and that *rpjF* mutants of *Xf* are hypervirulent and non-transmissible. Lack of transmissibility was linked to an inability of the *rpjF* mutant to form a biofilm in the insect foregut; while taken up by insects, the mutant strain is not retained. *Xf* strains that overproduce DSF produce disease symptoms in grape, but only at the site of inoculation and the cells do not move within the plant as do wild-type strains. Thus elevating DSF levels in plants should reduce movement of *Xf* in the plant and also reduce the likelihood of transmission by sharpshooters. We screened several collections of bacterial strains isolated from plants and identified bacterial strains that can interfere with *Xf* signaling both by producing large amounts of DSF, by degrading DSF, or by in some way interfering with recognition of DSF. When co-inoculated into grape with *Xf*, both DSF-producing strains and DSF degrading strains greatly reduced the incidence of disease in grape; DSF-producing strains consistently were the most effective in reducing disease. Given that DSF appears to mediate an attenuation of virulence in *Xf* we are in the process of transforming grape with the *rpjF* gene to enable DSF production in planta. Preliminary results indicate that transient expression of *rpjF* in *Nicotiana benthamiana* following infiltration with appropriate *Agrobacterium tumefaciens* strains resulted in high levels of DSF production, suggesting that it is likely that grape cells will produce DSF when transformed with the bacterial *rpjF* gene. Non-endophytic bacterial species were also established in high numbers inside grape leaves and petioles following spray application to plants with a high concentration of a silicon-based surfactant with a low surface tension, suggesting that it may be possible to produce protective compounds such as DSF in plants by a variety of bacteria.

## INTRODUCTION

Endophytic bacteria such as *Xylella fastidiosa* (*Xf*) colonize the internal tissues of the host, forming a biofilm inside the plant. A key determinant of success for an endophyte is the ability to move within the plant. We expect activities required for movement to be most successful when carried out by a community of cells since individual cells may be incapable of completing the feat on their own. Cells assess the size of their local population via cell-cell communication and coordinately regulate the expression of genes required for such processes. Our study aims to determine the role of cell-cell communication in *Xf* in colonization and pathogenicity in grapevines and transmission by the insect vector.

*Xf* shares sequence similarity with the plant pathogen *Xanthomonas campestris* pathovar *campestris* (*Xcc*) (7). In *Xcc*, expression of pathogenicity genes is controlled by the Rpf system of cell-cell communication, enabling a population of cells to launch a pathogenic attack in a coordinated manner (1). Two of the Rpf proteins, RpfB and RpfF, work to produce a diffusible signal factor (DSF) (1) which has recently been described as an alpha,beta unsaturated fatty acid (9):



As the population grows, the local concentration of DSF increases. Other Rpf proteins are thought to sense the increase in DSF concentration and transduce a signal, resulting in expression of pathogenicity factors (8). The *Xf* genome not only contains homologs of the *rpj* genes most essential for cell-cell signaling in *Xcc*, but also exhibits striking colinearity in the arrangement of these genes on the chromosome (2). We now have shown that *Xf* makes a molecule that is recognized by *Xcc*

but probably slightly different than the DSF of *Xcc*. Based on our knowledge of density-dependent gene regulation in other species, we predict the targets of Rpf regulation would be genes encoding extracellular polysaccharides, cellulases, proteases and pectinases necessary for colonizing the xylem and spreading from vessel to vessel. Similarly, we would expect the density-dependent genes to be expressed during the time when a population of *Xf* is ready to move into uncolonized areas.

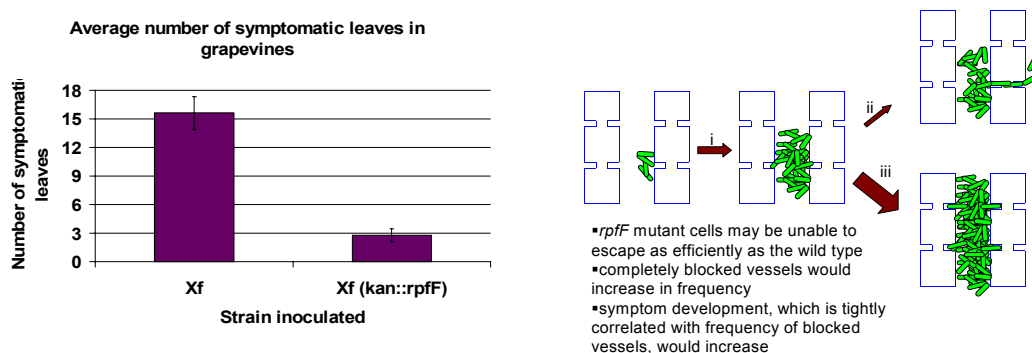
Other organisms can apparently interfere with the density-dependent behaviors of *Xf*. Several recent studies indicate that other organisms can disrupt or manipulate the cell-cell signaling system of bacteria (4, 5). We have found that several other bacterial species can both positively and negatively interact with the DSF-mediated cell-cell signaling in *Xf*, but until this study we did not know of the manner in which the interaction occurred nor whether such strains had the potential to affect the virulence of *Xf* in grape. In this period we have extensively investigated both the role of DSF-production by *Xf* on its behavior within plants and insects as well as the manner in which other bacterial strains affect such cell signaling and determined the extent to which other endophytes could modulate density-dependent behaviors and virulence in *Xf* by interfering with cell-cell signaling.

## OBJECTIVES

1. Determine role of signaling factors on virulence and transmissibility of *Xf*.
2. Identify degraders and producers of diffusible signaling factors used by *Xf*.
3. Perform Pierce's disease (PD) biocontrol tests on grapevines using DSF-interfering bacteria
4. Isolation of mutant strains of DSF-degrading and DSF activating bacteria that no longer interfere with cell-cell signaling in *Xf* to verify that disease control is linked to cell-cell signal interference
5. Creation of grapevines expressing gen4s conferring DSF-degradation and DSF-synthesis activities to test for PD resistance
6. Engineer grapevine endophytes such as *Alcaligenes xylosoxidans denitrificans* to express genes conferring DSF-degradation or DSF-synthesis activities and test whether the resulting transgenic endophytes are capable of disease control

## RESULTS

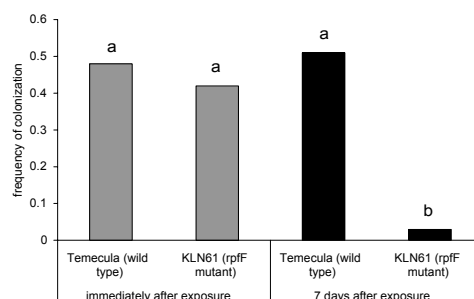
We have constructed a strain of *Xf* Temecula in which the *rpfF* gene, which is required for production of the signal in *Xcc*, is knocked out. This mutant was constructed using exchange of the wild-type allele for a deleted copy carrying an antibiotic resistance gene on a suicide plasmid. The *rpfF* mutant of *Xf* does not make DSF as determined using previously constructed "signal-sensing" strains of *Xcc* to determine DSF production by *Xf* and other bacterial strains. *rpfF* mutants strains were tested for their ability to infect and move within host plants and to cause Pierce's disease symptoms. The *rpfF* gene appears to play a role in modulating disease progress because the timing and severity of symptom development are greatly exacerbated in grapevines infected with *rpfF* mutants when compared to the wild type. We have investigated the mechanism behind these differences. We have found no detectable difference in populations or movement between the wild type and *rpfF* mutants, although our sampling methods would not be able to detect small increases in colonization if they existed. We hypothesize that *rpfF* mutants may be causing increased vessel blockage in the grapevine, leading to increased symptom expression. We have recently made a green fluorescent *rpfF* mutant to investigate the pattern of colonization by the mutant and compare it to that of the wild type. Importantly, when *rpfF* was over-expressed in *Xf* under the control of a high and constitutive promoter, the severity of disease in plants was greatly reduced (below). The *Xf* strain that overproduced DSF caused disease symptoms in grape, but only at the site of inoculation. The mutant cells did not move within the plant as did wild-type strains. These results all support our model that DSF regulates genes required for movement of *Xf* from colonized vessels.



Such results suggest that elevating DSF levels in plants should reduce movement of *Xf* in the plant.

We have tested transmissibility of the *rpfF* mutant strain by an insect vector. The *rpfF* mutant was virtually non-transmissible. This defect in transmissibility by the signaling-deficient mutant reveals the importance of cell-cell signaling in insect transmission. Leafhoppers fed on *rpfF*-infected plants ingested *rpfF* cells but were able to rapidly clear themselves whereas the wild type is never cleared.

*rpfF* mutants are taken up by insects but are rapidly cleared

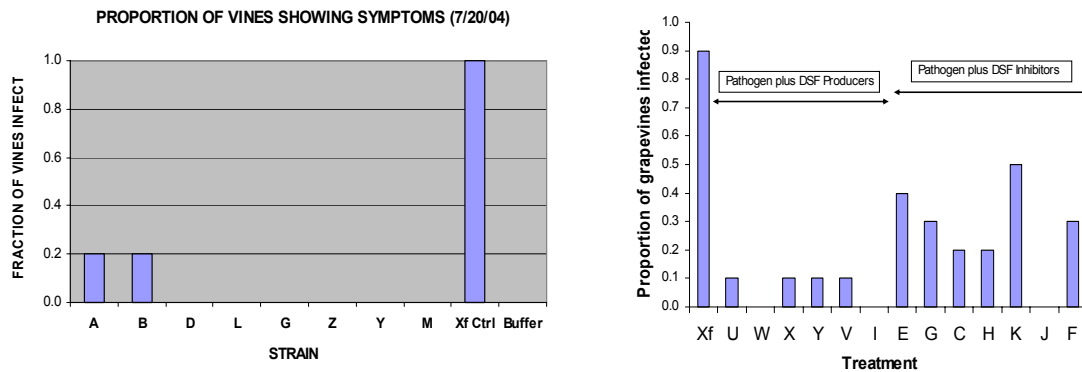


We have isolated a variety of bacteria from grapevines from vineyards affected by Pierce's disease as well as tomato and cruciferous crop plants infected with the signal-producing pathogens *Xanthomonas campestris* pv. *vessicatoria* and *Xcc*, respectively and tested them for their ability to interfere with cell-cell signaling in *Xf* in an assay using the signal-sensing strain described above. We found several strains that negatively affected signaling in *Xcc* while several strains were found to produce DSF. By adding purified DSF to either cell-free extracts of the strains with a negative influence on signaling or to whole cells we found that at least two mechanism of interference with signaling could be observed. Some strains such as strains C,E,G, H, and J are able to degrade DSF while other inhibitor strains did not do so, and apparently have another means of interfering with DSF perception by *Xcc*. The several strains that produced DSF were all identified as *Xanthomonas* species. We sequenced the 16S rRNA gene from these strains to determine their species identity.

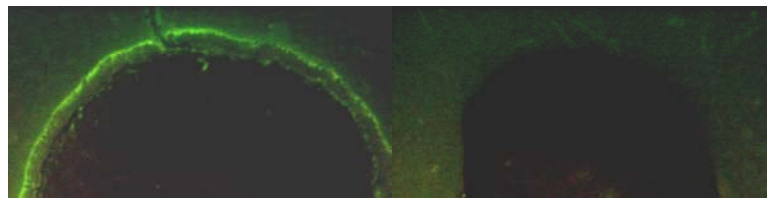
Strain	Genus	Origin	Mechanism of DSF Interference
A	<i>Paenibacillus</i>	Grape	Unknown inhibition
B	<i>Paenibacillus</i>	Grape	Unknown inhibition
C	<i>Pseudomonas</i>	Cabbage	Enzymatic digestion
D	<i>Staphylococcus</i>	Grape	Unknown inhibition
E	<i>Bacillus</i>	broccoli	Enzymatic digestion
G	<i>Pseudomonas</i>	Cabbage	Enzymatic digestion
H	<i>Pseudomonas</i>	Cabbage	Enzymatic digestion
J	<i>Pseudomonas</i>	Tomato	Enzymatic digestion
L	<i>Staphylococcus</i>	Grape	Unknown inhibition
I	<i>Xanthomonas</i>	Tomato	DSF production
U	<i>Xanthomonas</i>	Broccoli	DSF production
V	<i>Xanthomonas</i>	Broccoli	DSF production
W	<i>Xanthomonas</i>	Broccoli	DSF production
X	<i>Xanthomonas</i>	Broccoli	DSF production
Y	<i>Xanthomonas</i>	Tomato	DSF production
Z	<i>Xanthomonas</i>	Grape	DSF production

Interfering strain G, typical of strains that apparently degrade DSF, was subjected to transposon mutational analysis of the interfering activity. Several insertional mutations that block degradation of DSF have been identified and sequence analysis of the genes required for DSF degradation are being performed. We expect this analysis to reveal the identity of the gene responsible for the interfering activity. This gene can then be introduced into other organisms, such as plants.

To test the ability of bacteria that alter *Xf* signaling to alter the process of disease in plants, we co-inoculated grapevines with *Xf* and strains that either inhibit or activate cell-cell signaling in greenhouse studies. The incidence of Pierce's disease was greatly reduced by all of the signaling interfering strains that we tested. As we had expected, DSF-producing strains generally reduced disease severity more than did strains that interfered with signaling in *Xf*. These results were highly repeatable, having been observed in 2 separate experiments. We find these results to be very exciting in that they suggest that alteration of signal molecules within plants can have a profound effect on the disease process.



Given that DSF production by endophytes greatly reduces disease incidence and that DSF overproduction in *Xf* also reduces virulence, we have initiated studies to express *rpjF* in plants to achieve production of DFS in plants as a means of disease control. The *rpjF* gene from *Xf* as well as from *Xcc* was cloned into the plant transformation vector pCAMBIA to yield pKLN119. This plasmid carries a T-DNA that includes both hygromycin resistance and the *X. fastidiosa rpjF* gene driven by the CMV 35S promoter and followed by the NOS poly-A signal sequence. pKLN119 and the empty vector pCAMBIA1390 were electroporated into *Agrobacterium* strain GV3101. *Nicotiana benthamiana* plants were transiently transformed by infiltration with suspensions of *Agrobacterium* harboring T-DNA construct pKLN119 or pCAMBIA1390. Disks of infiltrated leaves were removed after two days, placed on KB agar plates and oversprayed with the DSF bioreporter strain 8525 (pKLN55). Substantial green fluorescence was observed in leaf disks of the plants into which pKLN119 was introduced (left), suggesting that *rpjF* conferred DSF production in *N. benthamiana*.



## CONCLUSIONS

Substantial data now show that cell-cell signaling plays a major role in the epidemiology and virulence of *Xf* and that disruption of cell signaling is a promising means of controlling Pierce's disease. Strikingly, *Xf* strains that cannot signal are also not transmissible by nor colonize an efficient insect vector. This result reveals an important and previously unappreciated connection between cell-cell signaling and transmission as well as the requirement for biofilm formation for transmission. These new findings will be helpful for those interested in targeting transmission as a means of disease control. We also found that mutants unable to signal are hypervirulent. Conversely, strains of *Xf* that overproduce DSF have low virulence and do not move within grape. This suggests that, it will be more efficient to elucidate and target *Xf*'s colonization strategies rather than traits predicted to contribute to virulence based on studies of other plant pathogens. We have identified bacterial strains that can interfere with *Xf* signaling. These strains proved very effective as protective agents for grapevines when co-inoculated with *Xf*. Both positive and negative interference with DSF signaling reduced disease in grape suggesting that signaling is normally finely balanced in the disease process; such a finely balanced process might be readily disrupted. Since in bacteria *rpjF* is sufficient to encode a synthase capable of DSF production, expression of DFS directly in plants is a attractive approach for disease control. Preliminary results are very encouraging that DSF can be made in plants. Alternatively, the use of various bacteria to express DSF implants may prove equally effective in altering *Xf* behavior and hence disease control.

## REFERENCES

- Barber, C. E., J. L. Tang, J. X. Feng, M. Q. Pan, T. J. Wilson, H. Slater, J. M. Dow, P. Williams, and M. J. Daniels. 1997. A novel regulatory system required for pathogenicity of *Xanthomonas campestris* is mediated by a small diffusible signal molecule. *Molecular Microbiology* 24:555-66.
- Dow, J. M., and M. J. Daniels. 2000. *Xylella* genomics and bacterial pathogenicity to plants. *Yeast* 17:263-71.
- Fry, S. M., and R. D. Milholland. 1990. Multiplication and translocation of *Xylella fastidiosa* in petioles and stems of grapevine resistant, tolerant, and susceptible to Pierce's disease. *Phytopathology* 80:61-65.
- Leadbetter, J. R., and E. P. Greenberg. 2000. Metabolism of acyl-homoserine lactone quorum-sensing signals by *Variovorax paradoxus*. *Journal of Bacteriology* 182:6921-6926.
- Manefield, M., R. de Nys, N. Kumar, R. Read, M. Givskov, P. Steinberg, and S. A. Kjelleberg. 1999. Evidence that halogenated furanones from *Delisea pulchra* inhibit acylated homoserine lactone (AHL)-mediated gene expression by displacing the AHL signal from its receptor protein. *Microbiology* 145:283-291.
- Severin, H. H. P. 1949. Transmission of the virus of Pierce's disease by leafhoppers. *Hilgardia* 19:190-202.

- Simpson, A. J. G., F. C. Reinach, P. Arruda, *et al.* 2000. The genome sequence of the plant pathogen *Xylella fastidiosa*. *Nature* 406:151-157.
- Slater, H., A. Alvarez-Morales, C. E. Barber, M. J. Daniels, and J. M. Dow. 2000. A two-component system involving an HD-GYP domain protein links cell-cell signalling to pathogenicity gene expression in *Xanthomonas campestris*. *Molecular Microbiology* 38:986-1003.
- Wang, Lian-Hui, He, Y., Gao, Y., Wu, J. E., Dong, Y.-H., He, C., Wang, S. X., Weng, L.-X., Xu, J.-L., Tay, L., Fang, and R. X., Zhang, L.-H. 2004. Cell-cell communication signal with cross-kingdom structural analogues. *Molecular Microbiology*. 51: 903-912.

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# SEASONAL POPULATION DYNAMICS OF GLASSY-WINGED SHARPSHOOTER EGG PARASITOIDS: VARIABILITY ACROSS SITES AND HOST PLANTS

## Project Leader:

Joseph G. Morse  
Dept. of Entomology  
University of California  
Riverside, CA 92521

## Cooperators:

David J. W. Morgan  
California Dept. of Food & Agriculture  
Mount Rubidoux Field Station  
Riverside, CA 92501

Jonathan M. Lytle  
Dept. of Entomology  
University of California  
Riverside, CA 92521

**Reporting Period:** The results reported here are from work conducted from July 12, 2004.

## ABSTRACT

The California Department of Food & Agriculture (CDFA) has a number of sites in southern California where they are releasing egg parasitoids of Glassy-winged Sharpshooter. To date, species released include *Gonatocerus ashmeadi*, *G. fasciatus*, *G. morrilli*, and *G. triguttatus*. Two South American *Gonatocerus* species are scheduled for release in 2005 (pending host specificity studies and release permits) and a strain of *Anagrus epos* from Minnesota may also be included in future releases (also pending such work). CDFA monitors for parasitoid establishment and population dynamics at release sites. This project is intended to complement and expand the scope of this monitoring with an eye towards improving our understanding of the benefit of releasing alternative parasitoid species and how well they are surviving, dispersing, and impacting GWSS populations.

## INTRODUCTION

One of CDFA's parasitoid release sites in southern California is Field 7H on the UC Riverside campus. A two-year field study in and around this release site was conducted to examine the temporal and host plant distribution of *Homalodisca* oviposition and associated egg parasitism (Al-Wahaibi 2004). In the current project, we plan to expand on this study and monitoring done by CDFA to attempt to improve our understanding of the population dynamics of endemic and released parasitoids in and around release sites. Although control programs appear to be effective at reducing Glassy-winged Sharpshooter populations, biological control is a more sustainable and environmentally friendly means of contributing to vector reduction and may have to suffice in much of California where chemical control is either impractical (e.g., urban areas) or economically unfeasible.

## OBJECTIVES

Monitor GWSS egg parasitoids in several areas in southern California in and around CDFA's parasitoid release sites and across several host plants.

## RESULTS

In the two-year study around CDFA's release site on the UC Riverside campus by Al-Wahaibi (2004), parasitism was due to a total of eight parasitoid species with *Gonatocerus ashmeadi*, *Ufens principalis* (previously *Ufens* A, Al-Wahaibi et al. 2005), *Ufens ceratus* (previously *Ufens* B), and *G. morrilli* being the most abundant. *Ufens* spp. were dominant on jojoba while on other plants, *Gonatocerus* species tended to dominate. Across all ten host plants sampled, ranked percent parasitism was *G. ashmeadi* (27.4%), *U. principalis* (19.8%), *U. ceratus* (2.9%), *G. morrilli* (2.1%), *G. incomptus* (0.4%), *G. novifasciatus* (0.3%), *G. triguttatus* (0.1%), and *G. fasciatus* (0.01%). Note, however, that these data may be biased by the proximity of nearby hosts harboring smoketree sharpshooter and high levels of *Ufens* spp. on jojoba.

We are in the process of expanding our sampling program outside of the UC Riverside campus.

## CONCLUSIONS

We are only 3 months into this project so it is too early to draw conclusions at present.

## REFERENCES

Al-Wahaibi, A.K., 2004. Studies on Two *Homalodisca* Species (Hemiptera: Cicadellidae) in Southern California: Biology of the Egg Stage, Host Plant and Temporal Effects on Oviposition and Associated Parasitism, and the Biology and Ecology of Two of their Egg Parasitoids, *Ufens* A and *Ufens* B (Hymenoptera: Trichogrammatidae). Ph.D. Dissertation, University of California, Riverside. 435 pp.

Al-Wahaibi, A.K., Owen, and J. G. Morse. 2005. Description and Behavioural Biology of Two *Ufens* Species (Hymenoptera: Trichogrammatidae), Egg Parasitoids of *Homalodisca* Species (Hemiptera: Cicadellidae) in Southern California. Bull. Entomol. Res. (Submitted).

#### **FUNDING AGENCIES**

Funding for this project was provided by the University of California Pierce's Disease Grant Program.

## TESTING TRANSGENIC GRAPEVINES FOR RESISTANCE TO PIERCE'S DISEASE

### Project Leaders:

Bruce I. Reisch  
Cornell University  
NYS Agric. Experimentation Station  
Geneva, NY 14456

Julie R. Kikkert  
Cornell University  
NYS Agric. Experimentation Station  
Geneva, NY 14456

Andrew Walker  
University of California  
Davis, CA 95616

### Cooperator:

José Vidal  
Cornell University  
NYS Agric. Experimentation Station  
Geneva, NY 14456

**Reporting period:** The results reported here are from work conducted from July 1, 2003 to September 30, 2004.

### ABSTRACT

About 80 'Chardonnay' lines transformed with the magainin-type antimicrobial peptide (AMP) genes, mag-2 and MSI99, and with a PGL class gene, were produced and about 40 were chosen for detailed analysis. Magainins are small peptides that inhibit growth of numerous bacteria and fungi. Some of these AMP-transgenic lines have improved resistance to tumorigenic strains of crown gall (*Agrobacterium vitis*). Other researchers have claimed that similar AMPs induce grapevine resistance to Pierce's disease (PD). The goal of our project was to characterize gene insertion, expression, and disease resistance in 'Chardonnay' grapevines harboring mag-2, MSI99, and a PGL class gene. These lines were shown to harbor between 1 and 5 copies of the foreign gene. RT-PCR testing showed that the genes are transcribed into RNA but efforts to directly detect peptide production in leaf tissues have been hampered by technical difficulties. We were able to show that some lines do produce the peptide, and that several have improved resistance to crown gall disease. The Walker Lab (UC Davis) has tested the same set of vines for resistance to PD. Symptom development was delayed in a few lines, which also showed improved lignification and re-growth following symptom development. Using an ELISA test to quantify the presence of the causal bacterium, 15 lines were classified as 'susceptible' while one line was classified as 'intermediate' in terms of resistance. Plants have been grafted to determine whether resistance (and/or transgene products) can be transmitted from a transformed rootstock to a non transformed scion cultivar. These tests are now underway.

### INTRODUCTION

Numerous genes involved in plant disease defense have been isolated (Punja 2001; Mourges et al. 1998). When disease resistance genes are introduced and expressed in transgenic plants, fungal and bacterial diseases have been greatly reduced (Mourges et al. 1998; Punja, 2001; Van der Biezen 2001). We have developed a set of transformed grapevines in which AMP genes are transcribed into RNA. About 80 'Chardonnay' lines transformed with the magainin-type genes, mag-2 and MSI99, and with a PGL class gene, were produced (Vidal et al. 2003). Magainins are small peptides with strong inhibitory activity against numerous bacteria and fungi (DeGray et al. 2001; Zasloff et al. 1988; Smith et al. 1998; Smith et al. 2001). The MSI99 peptide expressed in tobacco and banana was shown to be highly effective against several pathogens (Chakrabarti et al. 2003). In preliminary studies in 2002, some AMP-transgenic lines of 'Chardonnay' demonstrated improved resistance to tumorigenic strains of crown gall (*Agrobacterium vitis*), suggesting that these lines may harbor resistance to other bacterial diseases, as well.

Some AMP producing genes such as *Shiva-1* are effective against PD (Scorza and Gray, 2001) but the subject warrants further study. Scorza and Gray described a trial of two lines of 'Thompson Seedless' expressing the Shiva-1 peptide; both eventually succumbed to PD, but one had milder symptoms, which did not include the typical signs of marginal leaf burn when compared to the non-transformed control plant. However, data are not available in the literature to determine if mag-2, PGL, and MSI99 peptides are effective against *Xylella fastidiosa*. It is the purpose of the present project to study the potential resistance of our AMP-producing vines to PD.

### OBJECTIVES

1. Quantify the expression of AMPs (antimicrobial peptides) in transgenic 'Chardonnay' vines.
2. Evaluate resistance to Pierce's Disease among these transgenic vines.
3. Determine the extent to which an AMP transgenic rootstock can confer PD resistance to the scion.

### RESULTS

#### **Objective 1 - Quantify the Expression of AMPs (Antimicrobial Peptides) in Transgenic 'Chardonnay' Vines:**

Southern blots were used to determine the number of integration events in each positive line, as well as to determine which lines have full-length copies of the promoter + gene combination. Digested genomic DNA was separated by electrophoresis and visualized by chemiluminescence using digoxigenin-labeled probes. We tested 35 PCR positive lines and hybridization signals were detected in 34 lines. Between 1 and 5 AMP gene integration sites per line were detected. Hybridization banding

patterns differed among the lines, indicating independent transformation events. The entire non-fragmented promoter/signal peptide/AMP gene sequence was detected in twelve lines.

Transgene expression in leaves was quantified by ELISA. For the mag2 (23 amino acids) and MSI99 (22 amino acids) peptides, an antibody was developed (by Sigma-Genosys) that recognized an antigenic sequence common to both. In a series of preliminary ELISA tests (during 2003; methods per Li et al. 2001), low levels of peptide production were detected in 8 of 22 lines, in agreement with previous RT-PCR results. However we were unable to detect the peptide consistently, suggesting the methodology required some improvement. In spring 2004, a series of ELISA tests for peptide detection were carried out using very young leaves from greenhouse plants. Chardonnay lines transformed with either the gene for mag2 or for MSI99 production (ten of each), plus two non transformed lines, were assayed in three separate experiments. Despite rapid sample preparation, oxidation was an erratic problem among samples, and there were inconsistencies in the data collected. Among the ten lines expressing mag2, lines 167-3 and 167-9 were significantly different from the non transgenic controls. There were no significant differences in the ELISA assay among lines transformed with MSI99, however the highest ELISA readings were with lines 168-8 and 168-15. All four of these lines showed both mRNA transcription (via RT-PCR assays) and resistance to crown gall. Future attention was turned toward assaying peptide activity in bioassays to detect direct effects of plant extracts on bacterial growth.

***Crown Gall*** (cooperative work with Tom Burr; included here since it shows relative resistance to a bacterial disease among the same set of AMP transformed vines)

Crown gall resistance was assayed in two separate experiments. Thirty-one transgenic lines (4 plants/line) were inoculated with two different *Agrobacterium vitis* strains (TM4 and CG450). (Table 1 shows results for 16 lines.) Resistance was evaluated 60 d post inoculation based on gall size of 20 inoculation sites per line following a disease index (DI): 0 = no symptoms, 1 = small gall; 2 = medium gall; 3 = large gall; and 4 = very large gall. Among the lines tested, 6 harboring the mag2 gene, 5 with the MSI99 gene, 5 with the PGL gene and 5 with the combination of the mag-2 + PGL fusion gene showed statistically significant gall size reductions ( $P < 0.05$ ) compared to non-transformed controls (Table 1).

In the present study, a correlation was found between transcription level and resistance to the crown gall disease. We used a constitutive ubiquitin promoter from *Arabidopsis* to drive AMP gene expression. Although ubiquitin promoters are functional in *V. vinifera*, stronger promoters could be more useful for effective accumulation in plant tissue of small antimicrobial peptides. Finally, the level of resistance under potentially lower levels of inoculum in field conditions remains to be determined; greenhouse tests were done with high concentrations of inoculum.

#### ***Objective 2 - Evaluate Resistance to Pierce's Disease Among These Transgenic Vines:***

AMP-transgenic greenhouse-grown vines were tested for resistance to Pierce's disease. There were four groups chosen for testing, as shown in Table 1. Four lines were chosen to represent each of the four groups, and four vines of each line were tested. Vines were inoculated using the pin-prick needle inoculation technique of Hopkins (1980, 1984). Controls included two tissue-culture-produced non-transformed lines from the same set of experiments, plus a line of Chardonnay that was propagated from conventional cuttings. Transformed vines for this experiment were selected from among those with moderate to high rates of AMP gene transcription.

Results are not yet fully analyzed, but there is some variation for PD resistance among the 16 transgenic lines tested. All lines showed leaf symptoms of PD, but some lines had better cane lignification and new growth despite infection. Though symptom development was delayed in a number of lines, by about 3 months after collecting data on PD symptoms and samples for the ELISA assays, all vines had severe symptoms of PD or were dead. ELISA testing placed all lines but one in the 'susceptible' category, while one line harboring two AMP genes (319-13) was placed in the 'intermediate' category (Table 1). All control lines were classified as 'susceptible'. The two tissue cultured control lines did relatively well compared to conventionally propagated Chardonnay.

#### ***Objective 3 - Determine the Extent to Which an AMP Transgenic Rootstock can Confer PD Resistance to the Scion:***

Green grafting was used to connect five replicates of each transgenic line with non transgenic scions. Shoot growth on the scion 'Chardonnay' will be needle inoculated with the Stag's Leap *Xylella fastidiosa* strain. This work is still ongoing. About 70% of the grafted transgenic lines have been successfully produced and will soon be inoculated with *Xf*.

### **CONCLUSIONS**

Some indications of elevated resistance to PD are provided by the delays observed in symptom development and, for one line, the ELISA assays for bacterial concentration. However, after 3 months, all vines had severe symptoms of PD. Under natural field conditions with reduced inoculum concentrations, it is not yet known how these vines will perform. Use of AMP-transgenic vines to generate PD tolerant lines of important cultivars still seems to hold some promise, and warrants further testing. Confirmation of these initial results is still pending. The use of various means to target expression to the xylem may hold promise in future trials.

**Table 1.** Resistance to two bacterial diseases in AMP-transgenic ‘Chardonnay’ lines.

Line	Disease resistance <i>Crown Gall</i> <sup>a</sup>		Pierce's Disease		
	TM4	CG450	Mean cfu/ml (x 10 <sup>5</sup> ) ± std. error	PD Class <sup>b</sup>	Avg. visual symptoms ± std. error
<b><u>with Mag2</u></b>					
167-2	**	*	15.8±7.1	S	3.9±0.9
3	**	**	38.8±10.4	S	4.8±0.2
9	**	*	6.7±1.7	S	2.3±0.5
17	*		4.6±1.0	S	2.0±0.4
<b><u>with MSI99</u></b>					
168-8	*		16.3±6.6	S	3.3±1.1
15	**		8.1±1.8	S	1.3±0.8
32	**	*	5.3±1.2	S	2.4±0.8
37	*	*	15.5±7.2	S	2.6±1.1
<b><u>with PGL</u></b>					
315-5	**		20.2±6.1	S	4.7±0.7
17	*	*	12.0±5.9	S	3.4±1.0
19	*	*	15.2±2.6	S	4.4±0.4
20	**	**	11.4±1.7	S	2.5±0.9
<b><u>with mag2 + PGL</u></b>					
319-7	*		11.6±13.3	S	2.7±1.6
13	**	**	3.5±0.4	I	3.0±0.8
26	**	**	13.1±1.8	S	2.6±0.8
31	**		4.7±1.8	S	1.9±0.7
<b><u>controls:</u></b>					
NT8.1			8.2±2.4	S	3.1±0.6
NT8.2			7.5±2.6	S	2.5±0.7
Chardonnay			27.2±6.7	S	3.7±0.4

<sup>a</sup> Significantly resistant (\* P<0.05; \*\* P<0.01) when inoculated with TM4 or CG450 strains of *A. vitis*.

<sup>b</sup> PD Class

Resistant (R): ELISA mean cfu/ml <1 x 10<sup>5</sup>

Intermediate (I): ELISA mean cfu/ml >1 x 10<sup>5</sup> and sum of ELISA mean cfu/ml + Std Err <5 x 10<sup>5</sup>

susceptible (S): Sum of ELISA mean cfu/ml + Std Err >5 x 10<sup>5</sup>

Visual Symptoms Score

Genotypes with an average score of 2.0 or less can be considered resistant if ELISA values do not contradict.

Scores higher than 2.0 are indicative of susceptible genotypes

## REFERENCES

- Chakrabarti, A., T.R. Ganapathi, P.K. Mukherjee, and V.A. Bapat. 2003. MSI-99, a magainin analogue, imparts enhanced disease resistance in transgenic tobacco and banana. *Planta* 216:587-596.
- DeGray, G., K. Rajasekaran, F. Smith, J. Sanford, and H. Daniell. 2001. Expression of an antimicrobial peptide via the chloroplast genome to control phytopathogenic bacteria and fungi. *Plant Physiol.* 127:852-862.
- Hopkins, D.L. 1980. Use of pin-prick inoculation technique demonstrates variability in virulence of Pierce's disease bacterium. Pages 177-180 in: *Proc. of VII Conf. Viruses of Grapevine (ICVG)*, 8-12 September 1980, Niagara Falls, Canada.
- Hopkins, D.L. 1984. Variability of virulence in grapevine among isolates of the Pierce's disease bacterium. *Phytopathology* 74:1395-1398.
- Li, Z., S. Jayasankar, and D.J. Gray. 2001. An improved enzyme-linked immunoabsorbent assay protocol for the detection of small lytic peptides in transgenic grapevines (*Vitis vinifera*). *Plant Mol. Biol. Rep.* 19:341-351.
- Mourgues, F., M.N. Brisset, and E. Chevreau. 1998. Strategies to improve plant resistance to bacterial diseases through genetic engineering. *Trends in Biotechnology* 16:203-210.

- Punja, Z.K. 2001. Genetic engineering of plants to enhance resistance to fungal pathogens – a review of progress and future prospects. *Can. J. Plant Pathol.* 23: 216-235.
- Scorza, R. and D.J. Gray. 2001. Disease resistance in *Vitis*. United States Patent 6,232,528
- Smith, F.D., D.M. Gadoury, J.M. Van Eck, A. Blowers, J.C. Sanford, J. Van der Meij, and R. Eisenreich. 1998. Enhanced resistance to powdery mildew in transgenic poinsettia conferred by antimicrobial peptides. *Phytopathology* 88:S83.
- Smith, F., A.D. Blowers, J. Van Eck, and J.C. Sanford. 2001. Expression of magainin and PGL classes of antimicrobial peptide genes in plants, and their use in creating resistance to multiple plant pathogens. United State Patent 6,235,973.
- Van der Biezen, E.A. 2001. Quest for antimicrobial genes to engineer disease-resistant crops. *Trends Plant Sci.* 6:89-91.
- Vidal, J.R., J.R. Kikkert, P.G. Wallace, and B.I. Reisch. 2003. High-efficiency biolistic co-transformation and regeneration of ‘Chardonnay’ (*Vitis vinifera* L.) containing *npt-II* and antimicrobial peptide genes. *Plant Cell Rep.* 22:252-260.
- Zasloff, M., B. Martin, and H.C. Chen. 1988. Antimicrobial activity of synthetic magainin peptides and several analogues. *Proc. Natl. Acad. Sci.* 85:910-913.

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# LABORATORY AND FIELD EVALUATIONS OF IMIDACLOPRID (ADMIRE), THIAMETHOXAM (PLATINUM), AND ACETAMIPRID (ASSAIL) AGAINST THE GLASSY-WINGED SHARPSHOOTER

## Project Leaders:

Nick Toscano  
Dept of Entomology  
University of California  
Riverside, CA 92521

Frank Byrne  
Dept of Entomology  
University of California  
Riverside, CA 92521

Steve Castle  
Western Cotton Research Laboratory  
USDA-ARS  
Phoenix, AZ 85040

## Cooperators:

Mac Learned  
Bayer CropScience  
Paso Robles, CA

Carmen Gispert  
UC Cooperative Extension  
Indio, CA

Ben Drake  
Drake Enterprises  
Temecula, CA

**Reporting Period:** The results reported here are from work conducted from October 2003 to October 2004.

## ABSTRACT

Admire and Platinum applications were compared in Temecula vineyards. Although the uptake and residual persistence of both products was excellent, peak levels of Platinum within the xylem fluid of vines were almost 10-fold higher than Admire levels despite a lower application rate. In studies conducted in vineyards in the Coachella Valley, Admire uptake was considerably less efficient than that measured in Temecula vineyards. This could be rationalized by differences in soil properties between the two regions and the impact of irrigation practices. In soil column studies, Admire was shown to be more mobile in Coachella soils. Uptake was improved in vines that were under a strict irrigation regime that delivered water according to recommended vine requirements. Under these conditions, Admire within the xylem fluid attained recommended levels for GWSS suppression. In a preliminary evaluation of foliar applications of Assail, residues of acetamiprid, the active ingredient of this insecticide, were detected within the xylem fluid. Therefore, foliar applications may prove effective against GWSS both through contact and systemic activity.

## INTRODUCTION

In this report, we include data on the uptake and distribution of imidacloprid within grapevines in Temecula and Coachella Valley regions, focusing on the impact of soil type and irrigation on its rate of uptake and persistence within the plant over time. We also include laboratory-derived data from soil column studies, which demonstrate the impact of soil type on insecticide movement in soils from both regions. And, we evaluated two additional chemicals from the neonicotinoid insecticide class to determine their likely behavior under conditions in California vineyards.

## OBJECTIVES

1. Determine the impact of soil type and irrigation on the uptake and residual persistence of imidacloprid and thiamethoxam;
2. Determine the best combination of application rates and number of applications of imidacloprid and thiamethoxam in order to maximize and extend protection to vineyards;
3. Determine the absorption, distribution and residual persistence of foliar applications of acetamiprid within grapevines.

## RESULTS

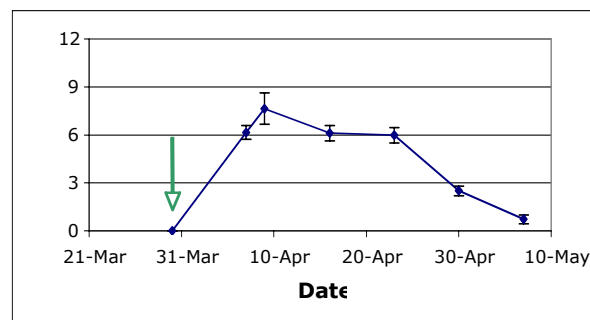
**Objective 1.** Determine the impact of soil type and irrigation on the uptake and residual persistence of imidacloprid and thiamethoxam.

### *Admire Applications in Coachella Valley Vineyards*

During the 2004 season, we continued to monitor the levels of imidacloprid in the xylem fluid of table grapevines treated with 16 fl oz/ acre in several vineyards in Coachella Valley. As in 2003, the peak titers of imidacloprid within the xylem sap of Coachella grapevines were extremely low and the overall persistence of the material was short-lived (Figure 1). In each of the vineyards tested, a single application of 16 fl oz/acre of Admire resulted in xylem fluid levels of imidacloprid of no more than 8 ppb, which is very close to the critical level required for protection against sharpshooter feeding.

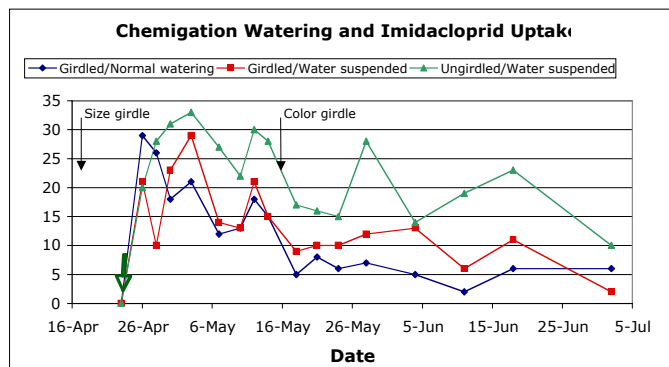
### *Impact of Irrigation and Girdling on Admire Uptake*

Over-watering is a potential problem for the effective use of Admire in Coachella vineyards, given the sandy soils. With this in



**Figure 1.** Uptake of Admire in a Coachella vineyard. The green arrow indicates the date of application of 16 fl oz/acre Admire. Each point is the mean  $\pm$  SEM of 24 vines

mind, we conducted an additional study in a vineyard where timing of irrigation to a Flame Seedless crop is determined from the crop coefficient. In addition to studying the effects of irrigation at the time of application, we also availed of the opportunity to study the impact of girdling on uptake into vines that were either size-girdled or not. We also examined the effect of withholding water from vines for two days subsequent to the application to test whether the Admire would ‘stick’ to the soil more effectively once it had been administered to the soil.

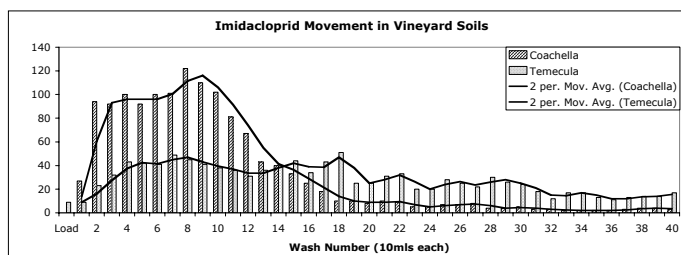


**Figure 2.** Uptake of Admire into girdled and ungirdled Flame Seedless grapevines. The green arrow indicates the Admire application date (16 fl oz/acre). Vines were either girdled (blue and red lines) or ungirdled (green line) for size. All vines were girdled for color on the same date. Water was suspended for two days after the Admire application in the red and green series.

therefore, that girdling can affect the efficiency of uptake. We intend to conduct further studies on the impact of girdling and irrigation on uptake during the 2005 season. One aspect of our work will be to try and define conditions for optimum uptake of Admire before the growers girdle their vines.

### Soil Column Studies

Laboratory-based soil column studies confirmed that the different soil properties in these areas could account for the differences in uptake (Figure 3). In these studies, the movement of imidacloprid through the Coachella soil column was more rapid, whereas the movement through the Temecula soil column was slower. This illustrates the impact that soil-type can have on insecticide availability. Under similar watering conditions, Admire is bound more tightly to the clay soil of the Temecula vineyard, compared with the sandier soils of the Coachella vineyard. If this mimics conditions in the field, then Admire will remain available for uptake into Temecula vines for a longer period. In reality, there are considerable differences between the two regions in the amounts of water used during irrigation. In Coachella Valley, where vast amounts of water are applied daily to each vine, it is possible that Admire is washed past the root zone before significant uptake can occur. In contrast, soils in Temecula vineyards can bind Admire more effectively, and with typically lower water volumes used during irrigation, more effective uptake of Admire is achieved.



**Figure 3.** Movement of imidacloprid through soil columns. Soil was collected from Temecula and Coachella vineyards where we conduct our uptake trials. The greater mobility of imidacloprid in Coachella soil is indicated by the more rapid recovery of imidacloprid.

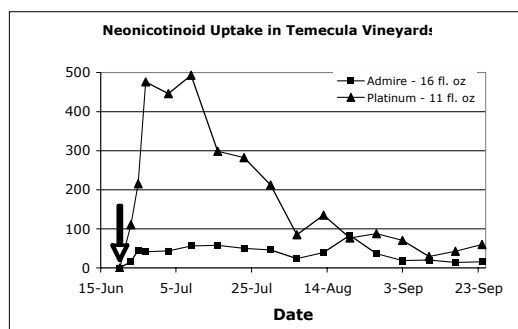
**Objective 2.** Determine the best combination of application rates and number of applications of imidacloprid and thiamethoxam in order to maximize and extend protection to vineyards.

### Admire and Platinum Applications in Temecula Valley Vineyards

In this component of the study, conducted in a Temecula Valley vineyard during 2003, we compared application rates of 16 fl oz/acre Admire and 11 fl oz/acre Platinum applied by drip chemigation (Figure 4). The titers of imidacloprid measured within the xylem fluid of vines treated with 16 fl oz/acre Admire were comparable with those measured in our previous trials conducted in Temecula vineyards (Toscano et al., 2003). This result establishes the consistent nature of Admire applications at this rate and hence the value of this product for GWSS management in vineyards. Despite using a lower Platinum application rate, peak titers of thiamethoxam within the xylem fluid exceeded those of imidacloprid by over 10-fold. And throughout the season, thiamethoxam levels were consistently higher than imidacloprid levels.



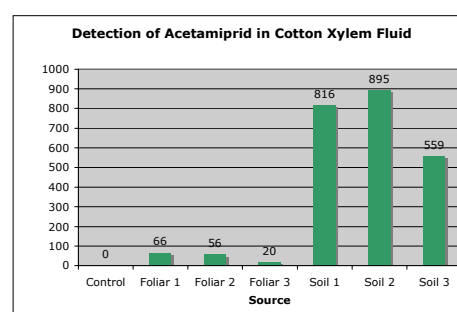
**Objective 3. Determine the Absorption, Distribution and Residual Persistence of Foliar Applications of Acetamiprid within Grapevines.**



**Figure 4.** Comparison of uptake profiles for Admire and Platinum applied at 16 fl oz and 11 fl oz/acre, respectively.

Acetamiprid is a third neonicotinoid of interest to growers and is recommended for foliar application to vines. It is reported to have systemic activity arising from translaminar movement of the chemical following application, as this will then be a more effective toxicant against the GWSS. Our studies were designed to determine the extent of the systemic activity of this material (applied as Assail). It is important to evaluate this aspect of acetamiprid behavior on grapes because it will determine the longevity of the product as a control measure for GWSS and PD transmission. The material will be short-lived if it does not penetrate into the plant, whereas translaminar movement is likely to enhance its efficacy by allowing for a more uniform distribution throughout the feeding zones of the GWSS. This could be important given the extremely low rates of application currently recommended for grapes, and could also compensate for less than complete coverage of the plant surface during application.

Assail was applied to the leaves of cotton plants by dipping individual leaves in insecticide solution. We avoided any contact between insecticide and the leaf petioles and main stem of the plants. After two weeks, the leaves were removed from the plant and the xylem fluid present in the main stem extracted and tested for the presence of acetamiprid using an ELISA detection method. We also treated plants by applying Assail to the soil, and the titers of acetamiprid present in the xylem of these plants was tested at two weeks post-treatment. Not surprisingly, we detected acetamiprid in the soil-treated plants (Figure 5). However, we also detected acetamiprid within the xylem of foliarly-treated plants, although the concentrations were considerably lower than in the soil-treated system. Nevertheless, our results indicate that the material had penetrated the leaf tissues and moved to the main stem where GWSS were most likely to feed. We are continuing with this approach using potted grapevines and citrus trees.



**Figure 5.** Acetamiprid levels in the xylem fluid of cotton treated foliarly (3 plants) and through the soil (3 plants) with Assail. Each bar represents the levels of insecticide measured in an individual plant.

## CONCLUSIONS

Our studies continue to provide growers with a better understanding of the behavior of Admire within vineyards in the southern California area. Certainly, there are significant differences between the Temecula and Coachella Valley vineyards and we are investigating the possible causes of these anomalies in our remaining study objectives. It seems clear for Temecula growers that a single application of 16 fl oz/acre can provide up to three months of protection from GWSS. An early season application of 16 oz/acre, followed by a later application at the same rate, would appear to be the most attractive option for achieving effective xylem sap levels of imidacloprid for protection against sharpshooters. GWSS are known to feed on vines throughout the year, including during the winter dormant phase, so by using the two-16 oz application strategy growers would be able to extend the window of protection well beyond that afforded to them with a single application of 32 oz/acre.

The titers of imidacloprid within the xylem fluid of vines in Coachella Valley vineyards treated at 16 oz/acre continue to be of concern. For two years now, we have monitored the effectiveness of the 16 fl oz rate and have found that most growers in this region are not receiving the same levels of protection for their vines as Temecula growers using the same rate. Our most recent study has shown the likely impact of watering on insecticide availability within the sandy soils of Coachella. If over-watering occurs it will compromise the effectiveness of Admire applications.

The results for uptake of imidacloprid and thiamethoxam are interesting. The uptake of imidacloprid is steady throughout the season, which should provide sustained protection to vines. In contrast, there is a rapid spike in thiamethoxam levels that takes concentrations to over 10-fold greater than imidacloprid. Further work is needed to determine suitable rates if this product is to be considered for use in vineyards.

**REFERENCES**

Toscano, N., Byrne, F., Castle, S., Bi J., Prabhaker, N., Learned, M., and C. Gispert. 2003. Laboratory and field evaluations of imidacloprid, thiamethoxam and acetamiprid against the glassy-winged sharpshooter on grapevines. Proceedings of the Pierce's Disease Research Symposium pp 315-317, Coronado Island Marriott Resort, San Diego, California. Dec 8-11, 2003.

**FUNDING AGENCIES**

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

## RIVERSIDE COUNTY GLASSY-WINGED SHARPSHOOTER AREA-WIDE MANAGEMENT PROGRAM IN THE COACHELLA AND TEMECULA VALLEYS

### Project Leaders:

Nick C. Toscano  
Dept. of Entomology  
University of California  
Riverside, CA 92521  
[nick.toscano@ucr.edu](mailto:nick.toscano@ucr.edu)

Raymond Hix  
Center for Viticulture  
Florida A & M University  
Tallahassee, FL 32309  
[Raymond.hix@famuedu](mailto:Raymond.hix@famuedu)

Carmen Gispert  
University of California  
Cooperative Extension  
Indio, CA 92201  
[cgispart@ucdavis.edu](mailto:cgispart@ucdavis.edu)

### Cooperators:

Thomas Perring  
Dept. of Entomology  
University of California  
Riverside, CA 92521  
[Thomas.perring@ucr.edu](mailto:Thomas.perring@ucr.edu)

John Snyder  
Riverside County  
Agricultural Commissioner's Office  
Riverside, CA 92502  
[jsnyder@co.riverside.ca.us](mailto:jsnyder@co.riverside.ca.us)

Robert Mulherin  
Riverside County  
Agricultural Commissioner's Office  
Riverside, CA 92502  
[rmulherin@co.riverside.ca.us](mailto:rmulherin@co.riverside.ca.us)

**Reporting Period:** The results reported here are from work conducted from October 2003 to September 2004.

### ABSTRACT

Riverside County has two general areas where citrus groves interface with vineyards; the Coachella and Temecula valleys. The Coachella valley with 12,000 acres of table grapes in proximity to 12,000 acres of citrus and the Temecula valley with 2,000 acres of wine grapes in proximity to 1,600 acres of citrus are vulnerable to Pierce's disease (PD), *Xylella fastidiosa*. The grapes in the Coachella and Temecula areas of Riverside County are in jeopardy because the glassy-winged sharpshooter (GWSS), the vector of the PD bacterium, builds up in adjacent citrus groves. Citrus is an important year-round reproductive host of GWSS in Riverside County, but also one that concentrates GWSS populations over the winter months during the time that grapes and many ornamental hosts are dormant. GWSS weekly monitoring in citrus and grapes began in March 2000 in Temecula valley and in 2003 in Coachella valley by trapping and visual inspections. Systemic insecticides such as Admire (imidacloprid), gave excellent control. In 2004 GWSS infestations in Temecula appear to be associated primarily with untreated tracts of vegetation such as organic citrus. Coachella valley GWSS populations have declined substantially relative to the pre-action levels due to insecticide applications.

### INTRODUCTION

The wine grape industry and its connecting tourist industry in Temecula valley generate \$100 million in revenue for the economy of the area. GWSS/PD caused a 30% vineyard loss and almost brought this wine-growing region to its knees. An area-wide GWSS management program initiated in the spring of 2000 saved the industry from a 100% loss. Only a continuation of an area-wide GWSS management program will keep the vineyards viable in Temecula. The table grape industry in the Coachella Valley is represented by 10,465 acres of producing vines, which generate fresh market grapes valued at an average of \$110 million annually. The GWSS was identified in the Coachella Valley in the early 1990's. Population increases of this insect in Coachella Valley in the last three years have increased the danger of PD occurrence in this area, as has occurred in similar situations in the Temecula and San Joaquin Valleys. In July 2002, the occurrence of *X. fastidiosa*, the PD bacterium, was found in 13 vines from two adjacent vineyards in the southeastern part of Coachella Valley. With this discovery, and the increasing GWSS populations, there was and is a real need to continue an area-wide GWSS/PD management program, to prevent an economic disaster to the work forces and connecting small businesses of Mecca, Thermal, Coachella, Indio, etc. that depend upon the vineyards for a big portion of their incomes. Only a continuation of an area wide GWSS/PD management program will keep the vineyards viable in Coachella. At present there are no apparent biological or climatological factors that will limit the spread of GWSS or PD. GWSS has the potential to develop high population densities in citrus. Insecticide treatments in citrus groves preceded and followed by trapping and visual inspections to determine the effectiveness of these treatments are needed to manage this devastating insect vector and bacterium. Approximately 2,135 acres of citrus in Riverside County were treated for the GWSS in February through June 2004 between a cooperative agreement with USDA-APHIS and the Riverside County Agricultural Commissioner's Office under the "Area-Wide Management of the Glassy-Winged Sharpshooter in the Coachella and Temecula Valleys".

### OBJECTIVES

1. Delineate the areas to be targeted for follow-up treatments to suppress GWSS populations in the Temecula and Coachella Valleys for 2004.
2. Determine the impact of the 2003 GWSS area-wide treatments to suppress GWSS populations in citrus groves and adjacent vineyards.
3. Determine the impact of a GWSS program on beneficial citrus insects, pest upsets and GWSS parasitoids.
4. Evaluate the biological and economic effectiveness of an area-wide insecticide program on GWSS.

## RESULTS AND CONCLUSIONS

The programs in Coachella and Temecula were dependent upon growers, pest management consultants, citrus and vineyard manager's participation. The areas encompass approximately 28,000 acres. Representatives of various agencies were involved in the program, they were as follows: USDA-ARS, USDA-APHIS, CDFA, Riverside County Agricultural Commissioner, UC-Riverside and grower consultants. Representatives of these agencies meet to review the program.

The GWSS/PD citrus groves and vineyards within the GWSS/PD management areas were monitored weekly to determine the need and effect of insecticide treatments on GWSS populations. Yellow sticky traps (7 x 9 inches) were used help determine GWSS population densities and dispersal/movement within groves and into vineyards. Based on trap counts and visual inspection, approximately 1,555 and 580 acres of citrus were treated in Coachella and Temecula, respectively for GWSS control. The following insecticides used and acres treated per insecticide are as follows: 1,935 acres with Admire at 36 ounces per acre; 40 acre; 40 acres with Baythroid (cyfluthrin) at 3.2 ounces per acre; 80 acres with Danitol (fenpropathrin) at 21.33 ounces per acre; and 80 acres with PyGanic (Pyrethrins) at 7 pints per acre.

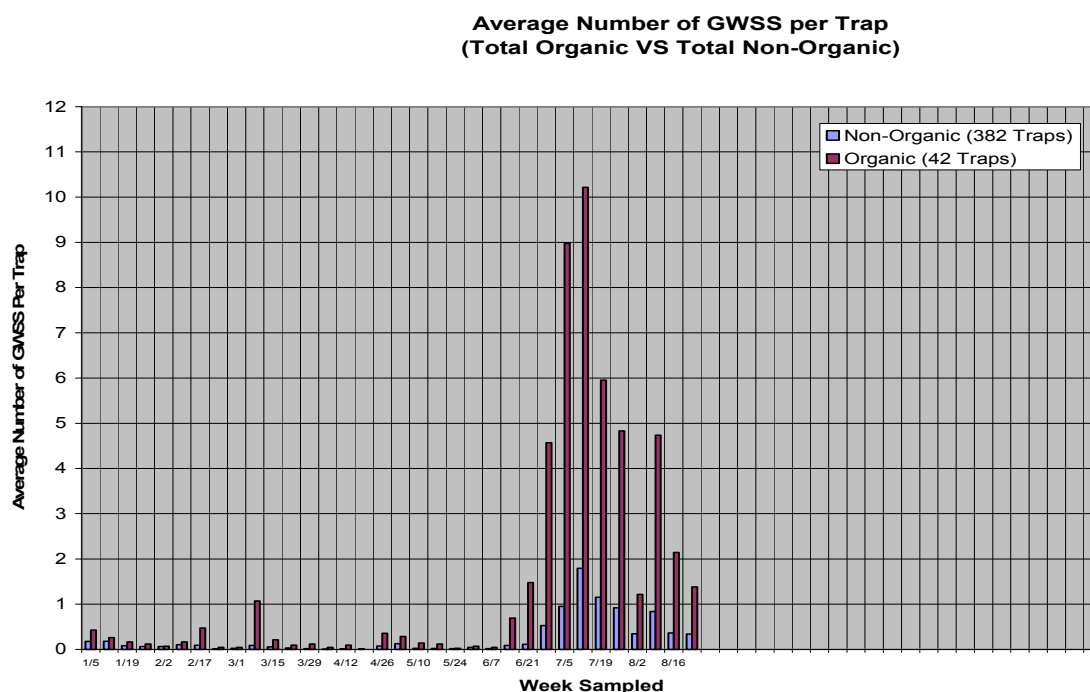
In 2004, high numbers of adult GWSS were caught on the yellow sticky traps in Temecula, with populations peaking in July reaching a total of almost 700 GWSS found (Figure 1). Figure 2 indicates that the highest numbers of GWSS, an average of 10 per trap, are trapped in organic orchards or citrus not treated with synthetic insecticides such as Admire. GWSS populations were almost non-existent in Coachella Valley with populations of the smoke-tree sharpshooter, *H. lacerta*, being the predominant species found on the sticky-traps (Figure 3).

For an area-wide GWSS management program to be successful with large acreages of citrus, a management program has to been initiated. Organic insecticides are not as effective as the neonicotinoid insecticides Admire and Assail or pyrethroids for controlling GWSS. Therefore, organic insecticides will have to be applied more frequently than its synthetic counterpart. Organic citrus groves pose challenges to area-wide GWSS management programs.

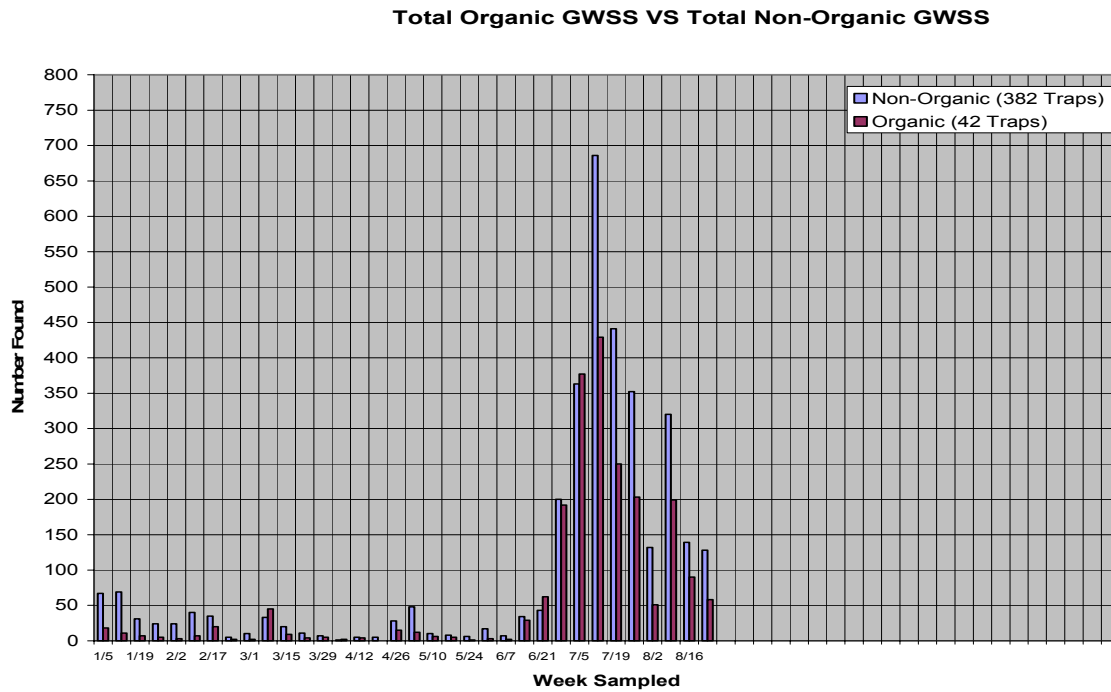
The programs in Coachella and Temecula were dependent upon growers, pest management consultants, and citrus and vineyard managers' participation. The areas involved encompass approximately 28,000 acres. Representatives of various agencies were involved in the program. They are as follows: USDA-ARS, USDA-APHIS, CDFA, Riverside County Agricultural Commissioner, UC-Riverside, and grower consultants. Representatives of these agencies meet as frequently as once a month to evaluate these Riverside County area-wide programs.

## FUNDING AGENCIES

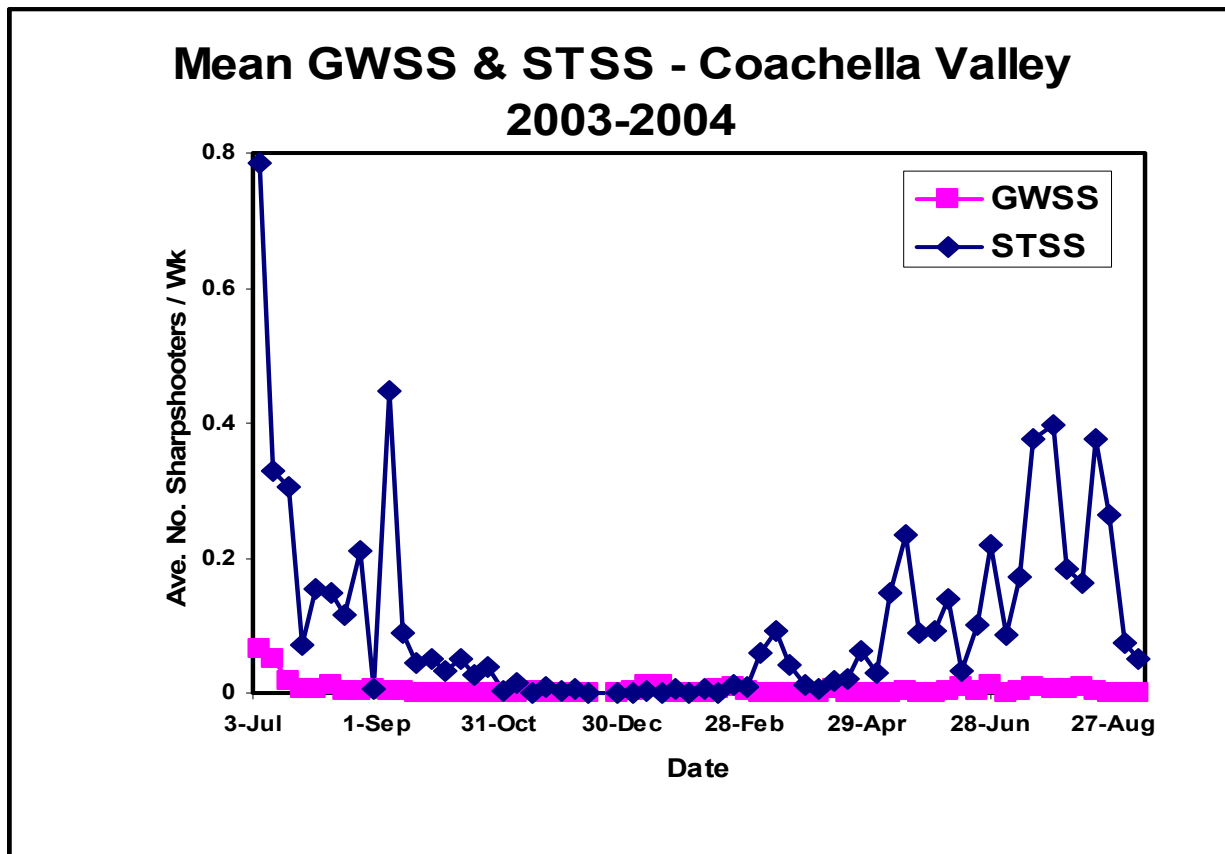
Funding for this project was provided by the USDA Animal and Plant Health Inspection Service and the California Department of Food and Agriculture.



**Figure 1.** The average number of glassy-winged sharpshooters trapped on yellow-sticky traps from January though August 2004, in Temecula valley.



**Figure 2.** The total number of glassy-winged sharpshooter trapped on yellow-sticky traps from January through August 2004, in Temecula valley.



**Figure 3.** Mean number of glassy-winged sharpshooters and smoke-tree sharpshooters trapped on yellow-sticky traps from July through August in Coachella valley.

## COMPATIBILITY OF INSECTICIDES WITH NATURAL ENEMIES OF THE GLASSY-WINGED SHARPSHOOTER

### Project Leaders:

Nick Toscano, Joseph G. Morse, & Nilima Prabhaker  
Dept. of Entomology  
University of California  
Riverside, CA 92521

Steven J. Castle & S. Naranjo  
USDA, ARS  
4135 E. Broadway Road  
Phoenix, AZ 85040

**Reporting Period:** The results reported here are from work conducted from July 2004 to September 2004.

### ABSTRACT

To enhance control of the glassy-winged sharpshooter (GWSS), relative toxicity of neonicotinoids, imidacloprid and thiamethoxam, and other conventional insecticides, chlorpyrifos, cyfluthrin and fenpropathrin, was examined to assess compatibility with parasitoids while being toxic to GWSS. Both imidacloprid and thiamethoxam when applied systemically through uptake in citrus leaves were found to be toxic to adult *Gonatocerus ashmeadi* and *Aphytis melinus*. However, systemic treatments of citrus and willow leaves infested with parasitized GWSS eggs did not impact the emergence of parasitoids significantly, suggesting they are relatively safe to the parasitoids while they are within the GWSS eggs. Fenpropathrin, a pyrethroid, was not toxic to *G. ashmeadi* for 3-4 days post-treatment while chlorpyrifos was quite toxic within 24 h to both *G. ashmeadi* and *A. melinus*. Future tests will measure the amounts of imidacloprid and thiamethoxam that are on the surface of citrus leaves affecting the survival of the parasitoids using ELISA. Based on these findings, our research will focus on understanding which chemicals are the most beneficial for maintaining a minimal impact on important parasitoids on citrus and grapes.

### INTRODUCTION

The glassy-winged sharpshooter (GWSS) is an important agricultural pest in California because it is a vector of several strains of the bacterium *Xylella fastidiosa* (*Xf*). The bacterium *Xf* causing Pierce's disease (PD) is transmitted to a number of host plants including grapes, citrus, olive and liquid amber trees (Wong et al. 2003). Citrus plays a large role in producing large populations of GWSS during spring and summer in southern California and dispersal to summer ornamentals contributing to the spread of *Xf*-diseased plants. Regional control programs that have targeted GWSS in citrus have proven highly successful in reducing GWSS densities in various parts of California. It is therefore essential to address the issue of GWSS management in citrus by adopting approaches that will ensure sustainable control.

The degree of compatibility among various control measures being used against GWSS is an important consideration in the development of sustainable management programs. Both field (Akey et al. 2001) and toxicological studies in the laboratory (Toscano et al. 2001) have shown that GWSS are extremely susceptible to both conventional and the newer neonicotinoid insecticides and can thus be used effectively to suppress GWSS populations. However, there is little information available on the long-term impact that chemical control measures against GWSS are having on its natural enemies and other predators and parasitoids, as well as on other pest species that attack citrus. To date, biological control has been critical in citrus IPM in California for many years, but is now threatened by the advent of new pests and greater use of insecticides to regain control. It is therefore essential to attain greater understanding of the impact of insecticide use for GWSS control on its natural enemies in citrus and how best it can be integrated with existing, successful management programs. The overall objective of this research proposal will be to help determine compatible management tactics by focusing on chemical control that is being used against GWSS and evaluating its impact upon several important biological control agents.

Several new insecticides in the neonicotinoid class of pesticides that have become important in agriculture during the last 4-5 years potentially play an important role in the control of GWSS due to their selectivity. Use of these more effective and selective insecticides have made it possible to target pest populations selectively while conserving their natural enemies (Naranjo 2001, Naranjo et al. 2004). With the use of neonicotinoid group of insecticides coupled with increasing knowledge of the predators and parasitoids important in the control of GWSS, a new citrus IPM program can be established that will provide effective and sustainable control. Therefore, to benefit the most from these selective insecticides in their potential adoption into IPM program for GWSS, we proposed to test their action against both the pest and their natural enemies. Imidacloprid, a systemic insecticide in this group, has been presumed to be safe for many natural enemies based on its systemic action. However, our preliminary results have shown a limited but detrimental impact on the *Gonatocerus ashmeadi*, suggesting a need for further investigations. Formulation of an insecticide can influence the uptake and penetration in a natural enemy based on the distribution of pesticide residues in the pest. This can lead to the question of any secondary effects on natural enemies with systemic insecticides. Research conducted to answer these questions will aid in identifying the most suitable insecticides that are specifically suited to the development of a locally adapted management system for GWSS. In addition to neonicotinoids, the impact on both the pest and natural enemies of select conventional insecticides that are also utilized for control of GWSS and other pests on citrus and grapes should be investigated to utilize the most ideal chemicals in GWSS management practices. Often the use of conventional insecticides is considered to be extremely negative in IPM practices due to adverse direct and indirect effects against biological control agents. If toxicity

assessment of conventional insecticides indicates preservation of natural enemies, it would allow a wider selection of products and responsible use patterns that may slow resistance development. The overall aim of this project is to evaluate the impact of a number of insecticides that are used in citrus and grapes on select natural enemies.

## OBJECTIVES

1. Monitor citrus orchards in Riverside, Ventura County and Coachella Valley to determine the relative abundance of select parasitoids and predators before and after treatment.
2. Evaluate select foliar and systemic GWSS pesticides used on citrus and grapes for their impact on GWSS egg parasitoids such as *Gonatocerus ashmeadi* and *G. triguttatus* as well as other parasitoids in the system such as *Aphytis melinus*.
3. Determine if honeydew produced by homopteran insects on citrus can be contaminated with systemic insecticides such as imidacloprid and thiamethoxam.
4. Determine the impact of imidacloprid and thiamethoxam residues within plant or within plant-feeding intoxicated insects, on the survivorship of *G. ashmeadi*, *G. triguttatus*, and *Aphytis melinus*.

## RESULTS

### Insects

*Gonatocerus ashmeadi* and *G. triguttatus* egg masses and adults were obtained from collections made in citrus and other hosts such as willow in Riverside. Insectary-reared shipments of *A. melinus* were obtained from Corona, California for tests conducted both in laboratory and field tests.

### Bioassay Techniques

Petri-dish bioassay: For foliar treatment, the petri dish bioassay technique was used to determine toxicity to two pyrethroids and two neonicotinoids. Leaf discs from citrus trees were dipped in various concentrations of acetamiprid, fenpropathrin and cyfluthrin and after allowing them to dry were placed in petri dishes with agar beds for exposure to the parasitoids for various intervals. At least 10 parasitoids per replicate, and 6 replicates per concentration were tested. A minimum of 5 concentrations per test along with a water control was evaluated. Toxicity of GWSS was also tested using this technique to compare the responses of the pest and its parasitoids. Additionally, *A. melinus* nymphs and adults (50-100) per petri dish were placed in the dishes with a drop of honey. Mortality assessment was made after 24 and 48 h.

### Field Collection of GWSS Egg-Infested Leaves

Willow and citrus leaves infested with GWSS eggs including parasitized eggs were also collected and subjected to the same treatment as above and placed in petri dishes to observe emergence and/or mortality. This test was conducted to examine toxicity of insecticides against parasitized eggs and the effect of insecticides on emergence or reduction due to mortality.

### Leaf-Uptake Systemic Bioassay

The systemic toxicity of imidacloprid and thiamethoxam was assessed using excised citrus leaves to allow uptake through the petioles directly into the leaf. The excised leaves were placed in serial dilutions of each compound contained in aquapiks for 24 hours. After 24 hours uptake time, treated leaves were placed in aquapiks containing water only. Parasitoids were exposed to each compound by enclosing them in clip cages attached to the treated leaves. Mortality counts were made after 24 and 48 hours.

### Objective 1.

Tests were initiated to determine the relative abundances of those natural enemies that are most active against GWSS in citrus orchards. Monitoring has not been initiated in Ventura County or Coachella Valley at the present time. Two methods were used to assess densities of the parasitoids *G. ashmeadi* and *G. triguttatus* as well as various predator species. Yellow sticky traps were posted at multiple locations within citrus orchards in Riverside for continuous monitoring of GWSS and natural enemies and changed once every week. Additionally, rates of parasitism by *G. ashmeadi* and *G. triguttatus* was evaluated by collecting citrus and willow tree leaves that were infested with GWSS egg masses and were placed in petri dishes with agar beds for incubation up to a week or longer. Numbers of GWSS nymphs and parasitoids emerging from each leaf were recorded throughout the season. Data collected from these traps shows that parasitoids were most abundant during mid-summer relative to GWSS activity. The parasitoids were also more abundant on willow leaves than citrus. In addition to *G. ashmeadi*, other species of parasites were also abundant in GWSS eggs on willow leaves. Predators were relatively few on the yellow traps and none were found using the petri dish technique. The numbers of *G. ashmeadi* decreased significantly from September in the leaf samples from both willow and citrus.

### Objective 2.

Relative toxicity of select insecticides to *G. ashmeadi* and *A. melinus* was assessed for imidacloprid, thiamethoxam, chlorpyrifos, cyfluthrin and fenpropathrin using petri dish for foliar applications and systemic uptake method for imidacloprid and thiamethoxam as described above. Data indicates that imidacloprid and thiamethoxam were toxic to the parasitoids even though the insects were exposed systemically and not directly. These results suggest that the two neonicotinoids were toxic to GWSS and did not preserve its beneficials as expected. Similar test results were obtained for *A. melinus* and *Encarsia* spp. also. As expected, chlorpyrifos was quite toxic to the beneficials. The predator, *Chrysoperla*, was not as susceptible to the systemic insecticides immediately but over time became more susceptible after 3-4 d of exposure.

More tests are in progress to address the reason for toxicity of the parasitoids to imidacloprid and thiamethoxam when applied systemically. Using ELISA, tests will be conducted to determine at what levels the systemic chemicals (imidacloprid and thiamethoxam) can be detected on the leaves after exposure to the two insecticides. Multiple testing methods will be used to evaluate if a specific dose of the two insecticides makes contact with the insect while they move around on the surface of the treated leaves.

#### **Objective 3.**

Tests have been initiated to examine if *A. melinus* is exposed to systemic insecticides while feeding on honeydew or through host feeding by adult parasitoids on intoxicated hosts on citrus. These tests will determine if there are residues of imidacloprid and thiamethoxam in honeydew when they are applied systemically. Initial tests have shown that *A. melinus* is extremely susceptible when caged on leaves of citrus trees that had been treated with the two insecticides over a year ago. Further tests will be conducted to determine at what levels the systemic chemicals (imidacloprid and thiamethoxam) can be detected in the honeydew produced by homopterans using ELISA kits.

#### **Objective 4.**

The potential for mortality caused by systemic insecticides that are in the plant tissue to parasitoids of GWSS, *Gonotocerus* spp. was examined by systemically treating willow leaves infested with parasitized GWSS egg masses. The impact on emergence of *Gonotocerus* spp. was not extensive compared to the toxicity of imidacloprid against the adult parasitoids that were mobile on plant surfaces treated systemically. Further tests will be conducted to determine directly the titers of either imidacloprid or thiamethoxam within the leaf tissue as well as in GWSS eggs using ELISA methods.

### **CONCLUSIONS**

Compatibility of select insecticides that are used for control of glassy-winged sharpshooter (GWSS) with representative parasitoids important in citrus was evaluated. Two systemic insecticides, imidacloprid and thiamethoxam were found to be toxic to the adult parasitoids of GWSS as well as to *A. melinus* in laboratory tests. However, systemic treatment with imidacloprid of willow leaves infested with parasitized GWSS eggs did not impact the emergence of parasitoids significantly suggesting that imidacloprid was relatively safe to *G. ashmeadi* and *G. triguttatus* during their development in the GWSS eggs. Results also indicated that chlorpyrifos was extremely toxic to the natural enemies while the pyrethroid, fenpropathrin, was not as toxic. Our results are expected to aid the development of pest management strategies based on the effective use of insecticides that selectively target pest species but are relatively harmless to GWSS parasitoids and other natural enemies present in citrus and grapes, thereby fostering enhanced biological control. Our research will focus on gaining an understanding of which chemicals are the most beneficial for maintaining a minimal impact on important parasitoids such as *G. ashmeadi* and *G. triguttatus*, as well as other parasitoids present on citrus and grapes. These data will also help to preserve IPM programs that have been established in different citrus-growing regions and help prevent pest flare-ups as a result of poor chemical control decision-making.

Toxicity of Select Insecticides to Parasitized GWSS Eggs				
Chemical	Total # Egg S. T	#Eggs D	#Emerg (parasites)	#Egg S. Exit holes <sup>a</sup>
Admire (10 ppm)	42	18	78	14
Lorsban	63	40	41	35
Danitol	30	10	156	19
Knack	31	16	120	16
Platinum (10 ppm)	64	44	39	19
Age of egg sacs unknown at the time of treatment				
<sup>a</sup> Parasitoid exit holes				



## REFERENCES

- Akey, D. H., T. J. Henneberry and N. C. Toscano. 2001. Insecticides sought to control adult glassy-winged sharpshooter. Calif. Agric. 55: 22-27.
- Naranjo, S.E. 2001. Conservation and evaluation of natural enemies in IPM systems for *Bemisia tabaci*. Crop Prot. 20: 835-852.
- Naranjo, S.E., P.C. Ellsworth, and J.R. Hagler. 2004. Conservation of natural enemies in cotton: Role of insect growth regulators for management of *Bemisia tabaci*. Biol. Cont. (*in press*).
- Toscano, N.C., S. J. Castle, J. Bi, F. Byrne, and N. Prabhaker. 2001. Laboratory and field evaluations of imidacloprid and thiamethoxam against GWSS on citrus and grapes. Pp. 121-122 in Symposium Proceedings, Pierce's Disease Research Symposium, Dec. 5-7, 2001. California Department of Food and Agriculture.
- Wong, F., D. A. Cooksey and H. S. Costa. 2003. Documentation and characterization of *Xylella fastidiosa* in landscape hosts. Pp. 131-135 in Symposium Proceedings, Pierce's Disease Research Symposium, Dec. 9-11. California Department of Food and Agriculture.

## FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

**PREPARING AND SUBMITTING FOR PUBLICATION A PICTORIAL, ANNOTATED KEY TO *GONATOCERUS* SPECIES AND OTHER GENERA AND SPECIES OF MYMARIDAE (HYMENOPTERA) – EGG PARASITOIDS OF *HOMALODISCA* SPP. AND OTHER PROCONIINE SHARPSHOOTERS IN NORTH AMERICA, WITH EMPHASIS ON THE SPECIES NATIVE OR INTRODUCED TO CALIFORNIA**

**Project Leader:**

Serguei V. Triapitsyn  
Dept. of Entomology  
University of California  
Riverside, CA 92521

**Cooperators:**

David J. W. Morgan  
California Dept. of Food and Agriculture  
Rubidoux Biological Center  
Riverside, CA 92504

Danel B. Vickerman  
Dept. of Entomology  
University of California  
Riverside, CA 92521

**Reporting Period:** The results reported here are from work conducted from July 1, 2004 to October 6, 2004.

**ABSTRACT**

We are at the first stage of this project (preparatory and curatorial work with voucher and other museum specimens).

**INTRODUCTION**

In North America, egg masses of proconiine sharpshooters (Hemiptera: Clypeorrhyncha: Cicadellidae: Cicadellinae: Proconiini), which are known or potential vectors of *Xylella fastidiosa*, are parasitized by various Mymaridae and Trichogrammatidae. An illustrated, annotated key to the genera and species of such Trichogrammatidae was already published (Triapitsyn 2003). However, a pictorial key, which could be used by non-taxonomists for recognition of the genera and species of Mymaridae, which are largely responsible for native biological control of proconiine sharpshooters in California, is lacking. In addition to the native mymarid parasitoids, several exotic species of *Gonatocerus* have been released recently in California as part of a classical biological control program against the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata* (Say), conducted by University of California, Riverside, CDFA, and USDA researchers. The proposed key will be a useful tool to distinguish them from other species of the same genus with similar host associations.

Moreover, because of the easy availability of proconiine sharpshooter eggs in California due to the establishment and outbreak of GWSS, there is a real possibility of non-intentional introductions of exotic egg parasitoids from countries in Central and South America. Recently, Dr. David Morgan (pers. comm.) reared from GWSS eggs in Irvine (Orange County) and submitted for identification an apparently undescribed species of *Gonatocerus* which is morphologically similar to some Central American taxa. This species has neither been known before from North America nor has been used in any biological control program. The proposed key (in which it will be described as a new taxon) will facilitate recognition of such species in California should they appear. Two new species of *Gonatocerus* from eastern Mexico ("*G. nr. ashmeadi*" and "*G. nr. morrilli*") will also be described, as they are perspective agents for introduction in California against GWSS.

Egg masses of the closely related *Homalodisca* and *Oncometopia* species, including GWSS, are parasitized by many species of *Gonatocerus*, all of which are members of the *ater* species group (Triapitsyn, 2002a, 2002b; Triapitsyn, Bezark & Morgan 2002). *Acropolynema* is the other mymarid genus that parasitizes eggs of *Homalodisca* (Triapitsyn, Hoddle & Morgan 2002). A species of *Anagrus* has been recently discovered as yet another genus of Mymaridae capable of parasitizing eggs of proconiine sharpshooters (Hoddle & Triapitsyn 2004). All mymarids, including *Gonatocerus*, are difficult to determine to species without expensive and labor-intensive preparation procedures such as critical point drying and slide-mounting, and their males are not easily recognizable at present. Consequently, a key to both sexes is being prepared that will be richly illustrated with color photographs of the main diagnostic characters as well as whole digital images of the species involved. Such a key will make it possible to correctly identify the most common mymarid parasitoids of *Homalodisca* and other proconiine sharpshooters directly in ethanol.

The material for this project has been accumulated mostly since 1997, with the beginning of surveys of egg parasitoids of GWSS in California and elsewhere in North America (Triapitsyn et al. 1998).

**OBJECTIVES**

This proposal has one main objective:

1. Prepare and submit for publication a pictorial, annotated key to mymarid egg parasitoids (mainly *Gonatocerus* spp.) of proconiine sharpshooters in North America, with emphasis on the species native or introduced to California. This key will also be made available on-line. The following experimental procedures are and will be used to accomplish this objective:

1. Preparatory and curatorial work. Egg parasitoids of GWSS and other proconiine sharpshooters were discovered through survey activities in California during 1996-2003 (Triapitsyn & Phillips 1996; Triapitsyn et al. 1998, Phillips et al. 2001, and S. Triapitsyn, unpublished) as well as elsewhere in the United States and Mexico conducted in 1997 (Triapitsyn et al. 1998), southeastern Texas in 1999 (Triapitsyn & Phillips 2000), Louisiana, northern Florida, southern Georgia, and southeastern Texas in 2000 (Morgan et al. 2000 and S. Triapitsyn, unpublished), throughout Florida and in Texas in 2001 (Triapitsyn and Hoddle 2001), in Georgia, Illinois, Louisiana, Kentucky, Mississippi, North Carolina, South Carolina, and Tennessee in 2002-2004 (Triapitsyn & Hoddle 2002; Hoddle & Triapitsyn 2003, 2004; Triapitsyn et al., 2003), and in Mexico during 1999-2003 (Triapitsyn & Phillips 2000, Triapitsyn, Bezark & Morgan 2002). Many of the discovered parasitoids were imported into UC Riverside quarantine, propagated, and their cultures turned over to CDFA and USDA researchers who released them in California under appropriate permits for biological control of GWSS. All mymarid parasitoids resulting from those surveys and laboratory and insectary colonies were preserved in 70% ethanol and deposited in the Entomology Research Museum at UC Riverside, California. Hundreds of such specimens of *Gonatocerus* and other Mymaridae are curated in the course of this project, and additional specimens will be made available from collections made in California by David Morgan. These are critically point-dried from ethanol, point- or card-mounted, labeled, and identified to species. Then representatives of each species (of both sexes) are selected, dissected, and slide-mounted. This is necessary for making digital photographs of the main distinguishing characters of each species.

2. Preparation of the illustrations and the key. After preparatory work with the specimens is complete, the three new species of *Gonatocerus* will be described taxonomically. Then we will take digital photographs (using AutoMontage digital technology) of the diagnostic features of each species as well as images of the habitus of females and males of each species. An illustrated key will be prepared, annotated with the diagnoses as well as data about host associations and distribution of all the species included in the key.

## RESULTS

Currently, we are at the first stage of this project (preparatory and curatorial work with voucher and other museum specimens). Principal Museum Preparator at the UC Riverside Entomology Research Museum (V. V. Berezovskiy) extracts egg parasitoids of proconiine sharpshooters from alcohol samples and point- and slide-mounts them. The specimens then get proper labels. All identifications are made by S. V. Triapitsyn. A genetic analysis of *G. "nr. ashmeadi"* from Mexico has been made by Danel Vickerman, and this species is ready to be described as a new taxon ( a separate publication is in preparation).

## CONCLUSIONS

Research resulting from this project will be of significant benefit to biological control (especially to the CDFA/PD Biological Control Program) specialists, ecologists, and vineyard supervisors that manage the Pierce's disease threat posed by GWSS. When completed, this key will enable even non-taxonomists to quickly identify both sexes of mymarid egg parasitoids of *Homalodisca* spp. in California, differentiate native vs. introduced species of *Gonatocerus*, provide information on candidate species of Mymaridae for introduction as part of biological control programs, facilitate surveys for assessing levels of egg parasitism of *H. coagulata* in the vineyards and orchards in California, and indicate all known host associations of the mymarid species important for native or classical biological control of glassy-winged sharpshooter and related species and genera of sharpshooters.

## REFERENCES

- Hoddle, M. S. & S. V. Triapitsyn. 2003. Searching for and collecting egg parasitoids of the Glassy-winged Sharpshooter in the central and eastern USA, pp. 261-262. *In: Proceedings of the Pierce's Disease Research Symposium*, December 8-11, 2003, Coronado Island Marriott Resort, Coronado, California. Organized by California Department of Food and Agriculture (compiled by M. A. Tariq, S. Oswalt, P. Blincoe, R. Spencer, L. Houser, A. Ba & T. Esser), Copeland Printing, Sacramento, California, 323 p.
- Hoddle, M. S. and S. V. Triapitsyn. 2004. Searching for and collecting egg parasitoids of the Glassy-winged Sharpshooter in the central and eastern USA. *In: Proceedings of the Pierce's Disease Research Symposium*, December 7-10, 2004.
- Morgan, D. J. W., S. V. Triapitsyn, R. A. Redak, L. G. Bezark, & M. S. Hoddle. 2000. Biological control of the glassy-winged sharpshooter: current status and future potential, pp.167-171. *In: [Proceedings] California Conference on Biological Control*, held July 11-12, 2000 at the historic Mission Inn, Riverside, California (M. S. Hoddle, Ed.), 205 pp.
- Phillips, P., S. Triapitsyn & M. S. Hoddle. 2001. Survey for egg parasitoids of glassy-winged sharpshooter in California, p. 95. *In: Proceedings of the Pierce's Disease Research Symposium*, December 5-7, 2001, Coronado Island Marriott Resort, San Diego, California. Organized by California Department of Food and Agriculture (compiled by M. A. Tariq, S. Oswalt & T. Esser), Copeland Printing, Sacramento, California. 141 pp.
- Triapitsyn, S. V. 2002a. Species-level taxonomy of Mymaridae (Hymenoptera): current status and implications for biological control of leafhoppers of economic importance, pp. 89-94. *In: Melika, G. & Thuróczy, C., editors. Parasitic wasps: evolution, systematics, biodiversity and biological control. Agroiinform*, Budapest, 480 pp.
- Triapitsyn, S. V. 2002b. Taxonomy and host associations of *Gonatocerus* spp. (Mymaridae) - egg parasitoids of proconiine leafhoppers. *Egg Parasitoid News* 14: 10.

- Triapitsyn, S. V. 2003. Taxonomic notes on the genera and species of Trichogrammatidae (Hymenoptera) - egg parasitoids of the proconiine sharpshooters (Hemiptera: Clypeorrhyncha: Cicadellidae: Proconiini) in southeastern USA. Transactions of the American Entomological Society 129 (2): 245-265.
- Triapitsyn, S. V., L. G. Bezark & D. J. W. Morgan. 2002. Redescription of *Gonatocerus atriclavus* Girault (Hymenoptera: Mymaridae), with notes on other egg parasitoids of sharpshooters (Homoptera: Cicadellidae: Proconiini) in northeastern Mexico. Pan-Pacific Entomologist 78 (1): 34-42.
- Triapitsyn, S. V. & M. S. Hoddle. 2001. Search for and collect egg parasitoids of glassy-winged sharpshooter in southeastern USA and northeastern Mexico, pp. 133-134. In: Proceedings of the Pierce's Disease Research Symposium, December 5-7, 2001, Coronado Island Marriott Resort, San Diego, California. Organized by California Department of Food and Agriculture (compiled by M. A. Tariq, S. Oswalt & T. Esser), Copeland Printing, Sacramento, California, 141 pp.
- Triapitsyn, S. V. & M. S. Hoddle. 2002. Search for and collect egg parasitoids of glassy-winged sharpshooter in southeastern USA and northeastern Mexico, pp. 94-95. In: Proceedings of the Pierce's Disease Research Symposium, December 15-18, 2002, Coronado Island Marriott Resort, San Diego, California. Organized by California Department of Food and Agriculture (compiled by M. A. Tariq, S. Oswalt, P. Blincoe & T. Esser), Digital Logistix, Sacramento, California, 177 p.
- Triapitsyn, S. V., M. S. Hoddle & D. J. W. Morgan. 2002. A new distribution and host record for *Gonatocerus triguttatus* in Florida, with notes on *Acmopolynema sema* (Hymenoptera: Mymaridae). Florida Entomologist 85 (4): 654-655.
- Triapitsyn, S. V., R. F. Mizell, III, J. L. Bossart & C. E. Carlton. 1998. Egg parasitoids of *Homalodisca coagulata* (Homoptera: Cicadellidae). Florida Entomologist 81 (2): 241-243.
- Triapitsyn, S. V., D. J. W. Morgan, M. S. Hoddle & V. V. Berezovskiy. 2003. Observations on the biology of *Gonatocerus fasciatus* Girault (Hymenoptera: Mymaridae), egg parasitoid of *Homalodisca coagulata* (Say) and *Oncometopia orbona* (Fabricius) (Hemiptera: Clypeorrhyncha: Cicadellidae). Pan-Pacific Entomologist 79 (1): 75-76.
- Triapitsyn, S. V. & P. A. Phillips. 1996. Egg parasitoid of glassy-winged sharpshooter. Citrograph 81 (9): 10.
- Triapitsyn, S. V. & P. A. Phillips. 2000. First host record of *Gonatocerus triguttatus* (Hymenoptera: Mymaridae) from eggs of *Homalodisca coagulata* (Homoptera: Cicadellidae), with notes on the distribution of the host. Florida Entomologist 83 (2): 200-203.

#### FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

## EVALUATION OF AN ANTIBACTERIAL PEPTIDE (CECROPIN A) AS A RESISTANCE AGENT IN PLANT XYLEM AGAINST *XYLELLA FASTIDIOSA*

### Project Leaders:

Donald S. Warkentin  
Dept. of Entomology  
University of California  
Davis, CA 95616

Shizuo George Kamita  
Dept. of Entomology  
University of California  
Davis, CA 95616

Bruce D. Hammock  
Dept. of Entomology  
University of California  
Davis, CA 95616

**Reporting Period:** The results reported here are from work conducted from July 1, 2004 to October 12, 2004.

### ABSTRACT

Cecropin A (Gudmundsson, et al., 1991) is a bactericidal peptide that is a potential source of resistance against *X. fastidiosa*, the causal bacterium of Pierce's disease. *In vitro* assays in our laboratory demonstrate that cecropin A is bactericidal against *X. fastidiosa*. We are utilizing the model plant, *Arabidopsis thaliana*, to evaluate the feasibility of using cecropin A expressed in transgenic plants as a resistance agent in plant xylem against *Xylella fastidiosa*. Because *X. fastidiosa* is a xylem-limited bacterium (Purcell and Hopkins, 1996; Hopkins, 1989), xylem-specific expression of cecropin A is required for effective resistance to Pierce's disease. To achieve our ultimate goal of producing grape rootstocks that confer Pierce's disease resistance to both the rootstock and the grafted scion, it will be necessary for cecropin A to circulate in the xylem vessels. To obtain expression of cecropin A in plant xylem, we are using a signal peptide sequence taken from a protein that naturally occurs in plant xylem. Glycine-rich proteins that are localized in plant xylem (Keller, et al., 1989; Morvan, et al., 2003) have been isolated and characterized (Sakuta and Satoh, 2000; Le Provost, et al., 2003). We are testing the rice glycine-rich protein signal peptide sequence, which confers vascular-specific expression in transgenic plants (Liu, et al., 2003), to determine if it will be effective to target cecropin A to plant xylem.

### INTRODUCTION

In early October 1999, University of California President Richard C. Atkinson established a task force of experts to help find solutions to Pierce's disease (Report of the Pierce's Disease Research and Emergency Response Task Force). Members of the Task Force believe that disease resistance, over the long term, offers the only sure protection for grapes from the ravages of the *X. fastidiosa* bacterium. The Task Force reported, however, that there is insufficient time for conventional plant breeding practices, which could take 20 years or more to breed resistance into grapes. Thus, the application of genetic engineering and other biotechnological techniques to insert disease-resistance genes into plants will be required.

By conventional breeding, it would be nearly impossible to produce varieties that are identical in every way to popular varieties such as Chardonnay, Pinot Noir, and Cabernet Sauvignon, except for one difference, resistance to Pierce's disease. Even by genetic engineering, it will be extremely difficult to produce Pierce's disease-resistance varieties that are identical to the currently most popular varieties in every other way but resistance. Thus, a strategy that is more likely to produce a consumer friendly result will be to generate a new rootstock that is Pierce's disease-resistant, and that also confers resistance to the grafted scion. This will require the identification and examination of Pierce's disease-resistance genes that can be introduced into rootstocks either by genetic engineering or by conventional breeding. These genes will have to encode gene products that will confer resistance not only to the rootstock, but also to the scion that is grafted onto it.

Cecropin A (Gudmundsson, et al., 1991) is a bactericidal peptide that is a potential source of resistance against *X. fastidiosa*, the causal bacterium of Pierce's disease. *In vitro* assays in our laboratory demonstrate that cecropin A is bactericidal against *X. fastidiosa*. To achieve our ultimate goal of utilizing cecropin A as one component of a multigenic approach to developing useful grape cultivars with resistance to *X. fastidiosa*, we will need to clear several preliminary hurdles. The first obstacle, demonstrating that cecropin A indeed has bactericidal activity versus *X. fastidiosa*, has already been accomplished *in vitro* in our laboratory.

The second step toward our goal will be to successfully express cecropin A in plants. Several bactericidal peptides, including cecropin B, have already been expressed in plants. Transgenic plants expressing cecropin B exhibit enhanced resistance to bacterial pathogens (Jaynes, et al., 1993). However, our *in vitro* studies indicate that cecropin A, rather than cecropin B, has greater bactericidal activity versus *X. fastidiosa*. Therefore, we are currently producing transgenic plants to express cecropin A. We are producing transgenic *A. thaliana* plants rather than immediately transforming grapevines because it is much faster to produce transgenic *A. thaliana* compared to the time that it would take to produce transgenic grapevines.

Expressing antibacterial peptides in grapevines is likely to provide resistance versus *X. fastidiosa* only if the expression products are correctly targeted to xylem tissues. Therefore, we are conducting several experiments to determine if the promoter, signal peptide, intron, antibacterial peptide, and transcription terminator combination that we have chosen will be successful to express active antibacterial peptide in the xylem of a model plant that is easily and rapidly transformable. Once we have succeeded in expressing an active antimicrobial peptide in xylem, then we will have the confidence to proceed to the next step in producing transgenic grapevines that are resistant against *X. fastidiosa*.

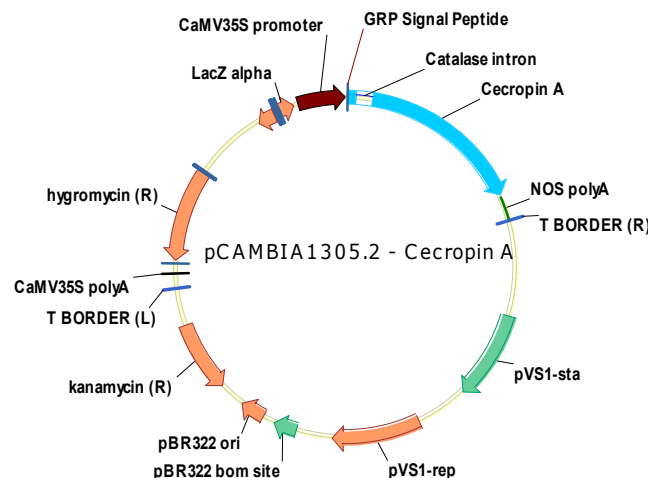
Another major hurdle will be to develop systems for expression of cecropin A in plant xylem. Because *X. fastidiosa* is a xylem-limited bacterium (Purcell and Hopkins, 1996; Hopkins, 1989), xylem-specific expression of cecropin A will be required for effective resistance to Pierce's disease. To achieve our goal of producing grape rootstocks that confer Pierce's disease resistance to both the rootstock and the grafted scion, it will be necessary for cecropin A to circulate in the xylem vessels. To obtain expression of cecropin A in plant xylem, we are using a signal peptide sequence taken from a protein that naturally occurs in plant xylem. Glycine-rich proteins that are localized in plant xylem (Keller, et al., 1989; Morvan, et al., 2003) have been isolated and characterized (Sakuta and Satoh, 2000; Le Provost, et al., 2003). We are testing the rice glycine-rich protein signal peptide sequence, which confers vascular-specific expression in transgenic plants (Liu, et al., 2003), to determine if it will be effective to target cecropin A to plant xylem.

## OBJECTIVES

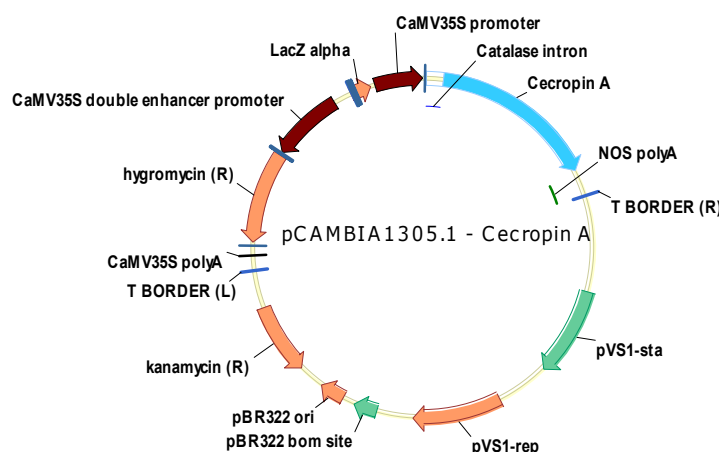
1. Express cecropin A in plants.
  - A. Utilize *Agrobacterium tumefaciens* to transform *Arabidopsis thaliana* with a cecropin A gene.
  - B. Assay putative transgenic plants via PCR, Southern blots, and western blots to verify the foreign gene insertion and expression.
2. Assay plants expressing cecropin A to determine their resistance versus plant pathogenic bacteria.
  - A. Assay transgenic plants expressing cecropin A versus the bacterial plant pathogen *Pseudomonas syringae* pv tomato (Pst).
  - B. Assay extracts of transgenic plants expressing cecropin A to determine if they have enhanced bactericidal activity versus *X. fastidiosa*.
3. Develop systems for xylem-specific expression of cecropin A in plants.
  - A. Transform *A. thaliana* with a gene construct linking a cecropin A gene with the rice glycine-rich protein signal peptide sequence (GRP).
  - B. Assay putative transgenic plants via PCR, Southern blots, and western blots to verify the foreign gene insertion and expression.
  - C. Assay xylem sap and xylem tissues from transgenic plants to determine if the GRP has directed the foreign protein to be expressed in xylem.

## RESULTS

For transformation of the model plant, *A. thaliana*, the sequence of the cecropin A gene from the Giant Silk Moth (*Hyalophora cecropia*) was codon modified (Perlak, et al., 1991) to conform with the codon usage of *A. thaliana*. Two plant transformation plasmid vectors, pCAMBIA1305.1 and pCAMBIA1305.2, (Roberts, et al., 1998) were modified by replacement of the GUSPlus gene with either a codon-modified mature cecropin A gene, or a codon-modified pro cecropin A gene (Figs. 1, 2). The plasmids pCAMBIA1305.1 and pCAMBIA 1305.2 are similar, except that the pCAMBIA1305.2 contains a rice glycine rich protein signal peptide. The plasmid construct with pCAMBIA1305.2 is made such that the cecropin A gene product will be fused with the rice glycine rich protein signal peptide sequence for targeting to the xylem. The transformation of *A. thaliana* with these plasmid constructs is currently in progress (Clough and Bent, 1998).



**Figure 1.** pCAMBIA 1305.1 plasmid vector containing Cecropin A gene.



**Figure 2.** pCAMBIA 1305.2 plasmid vector containing Cecropin A gene.

In developing a model system for testing foreign gene constructs as resistance agents versus *X. fastidiosa*, it will be very useful if a *X. fastidiosa* isolate can be identified that will develop systemic infections in *A. thaliana*. Therefore, we inoculated 50 *X. thaliana* seedlings with a Temecula grape isolate of *X. fastidiosa*. For negative controls, 25 seedlings were

inoculated with *Escherichia coli*, which is not expected to infect *A. thaliana*, and 25 seedlings were mock inoculated with PD3 bacteriological medium (Campanharo, et al., 2003). They will be assayed by ELISA and PCR for the development of systemic infections.

## CONCLUSIONS

Our recent laboratory results reveal that cecropin A has bactericidal activity *in vitro* against *X. fastidiosa*. Our project will test the antibacterial activity of cecropin A in transgenic plants. This is a preliminary step to determine if cecropin A would be a good candidate for expression in transgenic grapevines as a resistance agent versus *X. fastidiosa*. Expression strategies that result in xylem-specific expression are desirable. An antibacterial peptide produced in a grape rootstock that circulates in xylem would be ideal, because it would have great potential to allow the grafted scion to also be resistant to *X. fastidiosa*, without having to alter popular grape varieties.

We expect that cecropin A expressed in plants will have antibacterial activity. GRP signal peptide has already been demonstrated to direct foreign gene expression into vascular tissues (Liu, et al., 2003). We expect that the GRP signal peptide will also be effective for directing cecropin A into xylem, and that the cecropin A will circulate in xylem vessels. The confirmation of this hypothesis is one of the major goals of this project. If the answers are positive, then we would be confident to recommend cecropin A as an excellent candidate for production of grape rootstocks with resistance versus *X. fastidiosa*. For purposes of resistance management, we would recommend that cecropin A be one component of a multigenic resistance strategy.

## REFERENCES

- Campanharo, J.C., M.V.F. Lemos, and E.G.D. Lemos. *Growth optimization procedures for the phytopathogen Xylella fastidiosa*. Current Microbiology, 2003. 46(2): p. 99-102.
- Clough, S.J. and Bent. A.F. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. The Plant J. 16(6): 735-743.
- Gudmundsson, G.H., Lidholm, D.-A., Åsling, B., Gan, R., and Boman, H.G. 1991. The cecropin locus. J. Biol. Chem. 266: 11510-11517.
- Hopkins, D.L. 1989. *Xylella fastidiosa* xylem-limited bacterial pathogen of plants. Ann. Rev. Phytopathol. 27: 271-290.
- Jaynes, J.M., Nagpala, P., Destéfano-Beltrán, L., Huang, J. H., Kim, J.H. Denny, T., and Cetiner, S. 1993. Expression of a cecropin B lytic peptide analog in transgenic tobacco confers enhanced resistance to bacterial wilt caused by *Pseudomonas solanacearum*. Plant Sci. 89: 43-53.
- Keller, B., Templeton, M.D., and Lamb, C.J. 1989. Specific localization of a plant cell wall glycine-rich protein in protoxylem cells of the vascular system. Proc. Natl. Acad. Sci. USA 86: 1529-1533.
- Le Provost, G, Paiva, J., Pot, D., Brach, J., and Plomion, C. 2003. Seasonal variation in transcript accumulation in wood-forming tissues of maritime pine (*Pinus pinaster* Ait.) with emphasis on a cell wall glycine-rich protein. Planta 217: 820-830.
- Liu, Z.-Z., Wang, J.-L., Huang, X., Xu, W.-H., Liu, Z.-M., and Fang, R.-X. 2003. The promoter of a rice glycine-rich protein gene, *Osgrp-2*, confers vascular-specific expression in transgenic plants. Planta 216: 824-833.
- Morvan, C., Andème-Onzighi, C., Girault, R., Himmelsbach, D.S., Driouich, A., and Akin, D.E. 2003. Building flax fibres: more than one brick in the walls. Plant Physiology and Biochemistry 41: 935-944.
- Perlak, F.J., Fuchs, R. L., Dean, D.A., McPherson, S. L., and Fischhoff, D.A. 1991. Modification of the coding gene sequence enhances plant expression of insect control protein genes. Proc. Natl. Acad. Sci. USA 88: 3324-3328.
- Purcell, A.H. and Hopkins, D.L. 1996. Fastidious xylem-limited bacterial plant pathogens. Ann. Rev. Phytopathol. 34: 131-151.
- Roberts, C.S., Rajagopal, S., Smith, L.A., Nguyen, T.A., Yang, W., Nuroho, S., Ravi, K.S., Cao, M.-L., Vijayachandra, K., Patell, V., Harcourt, R., Dransfield, L., Desamero, N., Slamet, I., Keese, P., Kilian, A., and Jefferson, R.A. 1998. A comprehensive set of modular vectors for advanced manipulations and efficient transformation of plants by both *Agrobacterium* and direct DNA uptake methods. pCambia Vector release manual version 3.05. Cambia. Canberra, Australia.
- Sakuta, C. and Satoh, S. 2000. Vascular tissue-specific gene expression of xylem sap glycine-rich proteins in root and their localization in the walls of metaxylem vessels in cucumber. Plant Cell Physiol. 41(5): 627-638.

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## OPTIMIZATION OF ADMIRE APPLICATIONS IN NORTH COAST VINEYARDS

### Project Leaders:

Ed Weber  
UC Cooperative Extension  
Napa, CA 94559

Frank Byrne  
Dept. of Entomology  
University of California  
Riverside, CA 92521

Nick C. Toscano  
Dept. of Entomology  
University of California  
Riverside, CA 92521

**Reporting period:** The results reported here are from work conducted from March 2004 to October 2004.

### ABSTRACT

Four treatment regimes for Admire, a soil-applied imidacloprid insecticide, were evaluated in a North Coast Chardonnay vineyard in a cool site with a loam soil (15% clay content) and limited irrigation. None of the treatments resulted in consistent uptake or sustained concentrations of imidacloprid in xylem fluid at levels desired for control of blue-green sharpshooters or other insects. Our results suggest that limiting irrigation in order to manage vine growth and berry size for premium wine production may prevent the effective use of Admire in some cooler growing regions.

### INTRODUCTION

Admire insecticide (Bayer CropSciences) is widely used in grapes and citrus for control of the glassy-winged sharpshooter (GWSS) and to limit the spread of Pierce's disease (PD). It is a soil-applied product that delivers the active ingredient imidacloprid, a neonicotinoid insecticide that has been shown to be very effective against GWSS and other sucking insects (1). In northern California, Admire is sometimes used against populations of blue-green sharpshooters, the most common vector of PD in this region, as well as to treat for other pests.

Most research on uptake and persistence of imidacloprid in grapevines has been done in southern California in warm regions on soils with relatively low clay content (2, 3). In the heavy soils and cooler climates common in North Coast vineyards, there are questions about the best application strategy for Admire in order to ensure effective levels of imidacloprid in grapevines.

Admire is applied to vineyards through a drip system. It is recommended that it be applied to moist soils in order to enhance its downward movement into the root zone and its uptake into vines. Applying Admire early in the growing season (around budbreak) will meet the moist soil requirement. However, early in the season there is little canopy development, temperatures are cool, and therefore little uptake of water by the roots is occurring. In addition, because of the high rainfall and deep soils common in the North Coast conditions, grapevines have extensive root systems well outside the areas served by drip emitters. Early in the season, vines extract water from soil that is distant from the drip zone where Admire would be applied. Therefore, imidacloprid taken up early in the spring could be considerably diluted. Waiting until the soils dry down and the vines have established larger canopies should lead to more concentrated root activity in the drip zone and better uptake. However, the movement of Admire downward through the soil profile could be reduced due to the drier soil conditions.

To address these concerns and questions regarding the uptake of imidacloprid, we established a replicated field trial in the Carneros region of Napa County. Chardonnay vines on 101-14 rootstock were used. The vineyard was planted in 1994 with 8x5 foot spacing. The soil is a Haire loam with 15% clay content. There was one 4-liter/hour emitter for each vine. We chose this site because it presented a number of challenges to a soil-applied insecticide regime: cool growing conditions, non-sandy soil, extensive rooting beyond the drip zone and limited irrigation. These conditions are common among many North Coast vineyards.

### OBJECTIVE

1. Evaluate the uptake and sustained concentrations of imidacloprid in grapevines planted on clay soils in a cool region (Carneros) following different application regimes of Admire insecticide.

### RESULTS

Admire treatments were as follows:

- T1: 32 oz (full rate) in March
- T2: 16 oz in March / 16 oz in May
- T3: 32 oz in May
- T4: 16 oz in May / 16 oz in July

There were three replications of each treatment. Each replicate included three vine rows and extended the length of the vineyard block (136 vines). There was an untreated buffer row between each replicate. The vineyard was irrigated prior to each Admire application to ensure moist soil conditions, and water was applied for several hours after the injections were completed to move the material into the root zone. We checked each row to ensure that all drip emitters were functioning during our first application. Other than the irrigations made in conjunction with our treatment applications in March, May



and July, the only other irrigations during the growing season were a 4-hour application in May and a 1-hour application in July. An 8-hour post-harvest irrigation was made in September.

Xylem fluid samples were collected one week after the initial applications and continued every two weeks through September. Samples were collected only from the center row of each replicate. At each sampling, 200 microliters of xylem fluid were extracted from each of 8 vines within every replicate, and were kept separate. A different set of vines were sampled each week. Fluid extractions were made using a pressure bomb equipped with a large chamber that could accommodate shoots up to 18 inches in length. Samples were frozen on dry ice in the field and subsequently held in a freezer. Samples were shipped frozen overnight to UC Riverside and analyzed using a commercial ELISA detection kit (EnviroLogix, ME) (2).

None of the treatments resulted in effective uptake of imidacloprid. Average imidacloprid concentrations (N=24) for each treatment on each sampling date ranged from 0 to 3.71 ppb. 74% of the average values were less than 1 ppb. Ten ppb is considered to be a minimum threshold level for insecticidal activity in grapevines (3).

In many cases, all samples within a treatment had undetectable levels on a given week. During the course of the season, some vines were positive for imidacloprid in each replicate of each treatment, at both ends of the vineyard block. This indicates that Admire was effectively distributed with our injections. However, uptake by the vines was very limited. Some vines did take up some imidacloprid, but many apparently did not. Since different vines were sampled on each sampling date, our average values fluctuated on each sampling date. In no case was there evidence of sustained effective levels of imidacloprid in vines resulting from the treatments.

Table 1 summarizes our sampling results. It shows that the majority of samples were less than 4 ppb, the detection limit for this ELISA test. While Treatment 2 had the most samples with values greater than 4 ppb, these were just 12% of the samples. Only 4 out of the 1008 samples analyzed had imidacloprid levels that exceeded the minimum threshold for insecticidal activity of 10 ppb.

**Table 1:** Number of individual samples with imidacloprid concentrations in the ranges shown.

	<4 ppb	4-6 ppb	6-8 ppb	8-10 ppb	>10 ppb
<b>T1</b>	278	8	2	0	0
<b>T2</b>	253	20	9	4	2
<b>T3</b>	206	7	0	1	2
<b>T4</b>	211	4	1	0	0

## CONCLUSIONS

None of the Admire treatments resulted in consistent levels of imidacloprid in xylem fluid necessary to control blue-green sharpshooters or other sucking insects. The conditions presented by the vineyard used in this trial are not uncommon in many North Coast vineyards. The vines have widely distributed root systems in relatively deep, non-sandy soils with abundant soil moisture in the spring as a result of high winter rainfall. Evapotranspiration (and therefore water uptake) is less than in other growing regions due to smaller canopies and a cooler climate. Because of this, root activity is not concentrated in the drip zone until well into the growing season.

Nonetheless, our May and July applications did not result in any significant improvement to Admire uptake, at a time when more root activity within the drip zone was expected. Apparently, a key component limiting the uptake of Admire in this trial is the minimal level of irrigation.

In the production of North Coast grapes for premium wines, irrigation is often used sparingly in order to manage grapevine canopy development and to reduce berry size for optimal fruit and wine quality. This is especially true in the cooler growing regions and on heavier soils. In sites such as these, the uptake of Admire will be greatly limited and it is unlikely that it will have the desired insecticidal activity to justify its use.

## REFERENCES

1. Castle, S.J., Byrne, F.J., Bi, J.L., and Toscano, N.C. 2004. Spatial and temporal distribution of imidacloprid and thiamethoxam in citrus and impact on *Homalodisca coagulata* populations. Pest Management Science (In Press).
2. Toscano, N., et al. 2002. Laboratory and field evaluation of imidacloprid and thiamethoxam against GWSS on citrus and grapes. Proceedings, Pierce's Disease Research Symposium, San Diego, pp141-142.
3. Toscano, N., et al. 2003. Laboratory and field evaluation of imidacloprid and thiamethoxam and acetamiprid against the glassy-winged sharpshooter on grapevines. Proceedings, Pierce's Disease Research Symposium, San Diego, pp315-317.

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## EVALUATION OF BACTERICIDES AND GRAPEVINE ENDOPHYTES FOR MANAGEMENT OF PIERCE'S DISEASE

### Project Leader:

Bruce Kirkpatrick  
Dept. of Plant Pathology  
University of California  
Davis, CA 95616

### Cooperators:

Cheryl Whistler  
Dept. of Plant Pathology  
University of California  
Davis, CA 95616

Dick Hoenisch  
Dept. of Plant Pathology  
University of California  
Davis, CA 95616

Tina Winstrom  
Dept. of Environ. Sciences Policy & Mgmt.  
University of California  
Berkeley, CA 94720

Sandy Purcell  
Dept. of Environ. Sciences Policy & Mgmt.  
University of California  
Berkeley, CA 94720

Ed Weber  
UC Cooperative Extension Napa County  
Napa, CA 94559

### ABSTRACT

Results obtained in 2004 in our therapeutic and prophylactic bactericides were similar to those obtained in previous years. To date none of our prophylactic treatments using metallic plant microelements or inducers of systemic acquired resistance (SAR) provided protection against *Xylella fastidiosa* (*Xf*) infection in greenhouse or field studies. Results with the therapeutic treatments using microelements and 2 antibiotics were more encouraging and consistent with previously reported results. Pierce's disease (PD)-affected Merlot vines injected with zinc sulfate/oxide and streptomycin either 3 or 4 years ago had significantly less mortality than non-injected, PD control vines. We are continuing these studies in a Chardonnay vineyard located in Sonoma County; unfortunately another trial in Napa was unexpectedly removed by the grower. Six grapevine endophytes that exhibited antagonism to *Xf* *in vitro*, and moved greater than 8cm following pin-prick inoculations were inoculated into greenhouse grown vines in 2003. Six weeks later these vines were inoculated using *Xf*-infectious sharpshooters raised by Purcell's group at UC Berkeley. Results of symptom severity of these vines were reported in last year's Symposium. In spring, 2004 these vines were removed from the greenhouse and planted in the field at University of California, Davis. In October 2004 the vines were rated for mortality and PD symptoms. Only one *Xf*-inoculated, positive control remained alive. None of the 10 vines inoculated with one of the endophytes had any PD symptoms; other endophytes varied from 0 to 8 healthy appearing vines. These vines will be tested for the presence of *Xf* using PCR and the most promising candidates will be re-tested in the field in 2005. Another set of *Pseudomonas* endophytes that exhibited movement *in planta* and *in vitro* antagonism were inoculated into grapevines growing in the greenhouse. Symptom development and the presence of the endophyte and *Xf* in the vines will be determined in November 2004.